

**POLYPEPTIDES HAVING A FUNCTIONAL DOMAIN OF INTEREST  
AND METHODS OF IDENTIFYING AND USING SAME**

This application is a continuation-in-part of co-  
5 pending U.S. Patent Application Serial No. 08/417,872 filed  
April 7, 1995, the entire contents of which are incorporated  
herein by reference.

**1. Introduction**

10           The present invention is directed to polypeptides  
having a functional domain of interest or functional  
equivalents thereof. Methods of identifying these  
polypeptides are described, along with various methods of  
their use, including but not limited to targeted drug  
15 discovery.

**2. Background of the Invention**

Combinatorial libraries represent exciting new tools  
in basic science research and drug design. It is possible  
20 through synthetic chemistry or molecular biology to generate  
libraries of complex polymers, with many subunit permutations.  
There are many guises to these libraries: random peptides,  
which can be synthesized on plastic pins (Geysen et al., 1987,  
J. Immunol. Meth. 102:259-274), beads (Lam et al., 1991,  
25 Nature 354:82-84) or in a soluble form (Houghten et al., 1991,  
Nature 354:84-86) or expressed on the surface of viral  
particles (Cwirla et al., 1990, Proc. Natl. Acad. Sci. USA  
87:6378-6382; Kay et al., 1993, Gene 128:59-65; Scott and  
Smith, 1990, Science 249:386-390); nucleic acids (Ellington  
30 and Szostak, 1990, Nature 346:818-822; Gao et al., 1994, Proc.  
Natl. Acad. Sci. USA 91:11207-11211; Tuerk and Gold, 1990,  
Science 249:505-510); and small organic molecules (Gordon et  
al., 1994, J. Med. Chem. 37:1385-1401). These libraries are  
very useful in mapping protein-protein interactions and  
35 discovering drugs.

Phage display has become a powerful method for  
screening populations of peptides, mutagenized proteins, and

cDNAs for members that have affinity to target molecules of interest. It is possible to generate  $10^8$ - $10^9$  different recombinants from which one or more clones can be selected with affinity to antigens, antibodies, cell surface receptors, protein chaperones, DNA, metal ions, etc. Screening libraries is versatile because the displayed elements are expressed on the surface of the virus as capsid-fusion proteins. The most important consequence of this arrangement is that there is a physical linkage between phenotype and genotype. There are several other advantages as well: 1) virus particles which have been isolated from libraries by affinity selection can be regenerated by simple bacterial infection, and 2) the primary structure of the displayed binding peptide or protein can be easily deduced by DNA sequencing of the cloned segment in the viral genome.

Combinatorial peptide libraries have been expressed in bacteriophage. Synthetic oligonucleotides, fixed in length, but with multiple unspecified codons can be cloned into genes III, VI, or VIII of bacteriophage M13 where they are expressed as a plurality of peptide:capsid fusion proteins. The libraries, often referred to as random peptide libraries, can be screened for binding to target molecules of interest. Usually, three to four rounds of screening can be accomplished in a week's time, leading to the isolation of one to hundreds of binding phage.

The primary structure of the binding peptides is then deduced by nucleotide sequencing of individual clones. Inspection of the peptide sequences sometimes reveals a common motif, or consensus sequence. Generally, this motif when synthesized as a soluble peptide has the full binding activity. Random peptide libraries have successfully yielded peptides that bind to the Fab site of antibodies (Cwirla et al., 1990, Proc. Natl. Acad. Sci. USA 87:6378-6382; Scott and Smith, 1990, Science 249:386-390), cell surface receptors (Doorbar and Winter, 1994, J. Mol. Biol. 244:361-369; Goodson et al., 1994, Proc. Natl. Acad. Sci. USA 91:7129-7133), cytosolic receptors (Blond-Elguindi et al., 1993, Cell 75:717-

728), intracellular proteins (Daniels and Lane, 1994, J. Mol. Biol. 243:639-652; Dedman et al., 1993, J. Biol. Chem. 268:23025-23030; Sparks et al., 1994, J. Biol. Chem. 269:23853-23856), DNA (Krook et al., 1994, Biochem. Biophys. Res. Comm. 204:849-854), and many other targets (Winter, 1994, Drug Dev. Res. 33:71-89).

Most vital cellular processes are regulated by the transmission of signals throughout the cell in the form of complex interactions between proteins. As the study of signal transduction, or the flow of information throughout the cell, has broadened and matured, it has become apparent that these protein-protein interactions are often mediated by modular domains within signalling proteins. Src, both the first proto-oncogene product and the first tyrosine kinase discovered (Taylor and Shalloway, 1993, Current Opinion in Genetics and Development 3:26-34), is the prototypic modular domain-containing protein.

Src is a protein tyrosine kinase of 60 kilodaltons and is located at the plasma membrane of cells. It was first discovered in the 1970's to be the oncogenic element of Rous sarcoma virus, and in the 1980's, it was appreciated to be a component of the signal transduction system in animal cells. However, since the identification of viral and cellular forms of Src (i.e., v-Src and c-Src), their respective roles in oncogenesis, normal cell growth, and differentiation have not been completely understood.

In addition to its tyrosine kinase region (sometimes called a Src Homology 1 domain), Src contains two regions that have been found to have functionally and structurally homologous counterparts in a large number of proteins. These regions have been designated the Src Homology 2 (SH2) and Src Homology 3 (SH3) domains. SH2 and SH3 domains are modular in that they fold independently of the protein that contains them, their secondary structure places N-and C-termini close to one another in space, and they appear at variable locations (anywhere from N-to C-terminal) from one protein to the next (Cohen et al., 1995, Cell 80:237-248). SH2 domains have been

well-studied and are known to be involved in binding to phosphorylated tyrosine residues (Pawson and Gish, 1992, Cell 71:359-362).

The Src-homology region 3 (SH3) of Src is a domain  
5 that is 60-70 amino acids in length and is present in many cellular proteins (Cohen et al., 1995, Cell 80:237-248; Pawson, 1995, Nature 373:573-580). Within Src, the SH3 domain is considered to be a negative inhibitory domain, because c-Src can be activated (i.e., transforming) through mutations in  
10 this domain (Jackson et al., 1993, Oncogene 8:1943-1956; Seidel-Dugan et al., 1992, Mol Cell Biol 12:1835-1845).

To deduce the binding specificity of the Abl SH3 domain, a group led by David Baltimore screened cDNA libraries with radiolabeled GST-Abl SH3 fusion protein and identified  
15 two binding cDNA clones (Cicchetti et al., 1992, Science 257:803-806). Both clones encoded proteins with proline rich regions that were later shown to be SH3 binding domains.

Subsequently, others have screened combinatorial peptide libraries and identified peptides that bound to the  
20 Src SH3 domain (Yu et al., 1994, Cell 76:933-945; Cheadle et al., 1994, J. Biol. Chem. 269:24034-24039). Using the SH3 domain of Src, Sparks et al., 1994, J. Biol. Chem. 269:23853-23856 screened phage-display random peptide libraries and identified a consensus peptide sequence that binds with  
25 specificity and high affinity to the Src SH3 domain.

The consensus from these various studies is that the optimal Src SH3 peptide ligand is RPLPPLP (SEQ ID NO:45). Recently, the structures of the peptide-SH3 domain complexes have been deduced by NMR and the peptides have been shown to  
30 bind in two possible orientations with respect to the SH3 domain (Feng et al., 1994, Science 266:1241-1247; Lim et al., 1994, Nature 372:375-379).

Since SH3 domains have been found to have such important roles in the function of crucial signalling and  
35 structural elements in the cell, a method of identifying proteins containing SH3 regions is of great interest. In this regard, it is important to note that such a method is

unavailable because of the low sequence similarity of modular functional domains, including SH3. See, e.g., Figure 6, which illustrates the minimal primary sequence homology among various known SH3 domains.

5           Sequence homology searches can potentially identify known proteins containing not yet recognized functional domains of interest, however, sequence homology generally needs to be >40% for this procedure to be successful.

Functional domains generally are less than 40% homologous and  
10 therefore many would be missed in a sequence homology search. In addition, homology searches do not identify novel proteins; they only identify proteins already defined by nucleotide or amino acid sequence and present in the database.

Another approach is to use hybridization techniques  
15 using nucleotide probes to search expression libraries for novel proteins. This method would have limited applicability to finding novel proteins containing functional domains due to the low sequence homology of the functional domains.

Methods for isolating partner proteins involved in  
20 protein-protein interactions have generally focused on finding a ligand to a protein that has been found and characterized. Such approaches have included using anti-idiotypic antibodies that mimic the known protein to screen cDNA expression libraries for a binding ligand (Jerne, 1974, Ann. Immunol.  
25 (Inst. Pasteur) 125c:373-389; Sudol, 1994, Oncogene 9:2145-2152). Skolnick et al., 1991, Cell 65:83-90 isolated a binding partner for PI3-kinase by screening a cDNA expression library with the <sup>32</sup>P-labeled tyrosine phosphorylated carboxyl terminus of the epidermal growth factor receptor (EGFR).

30           An easy method for isolating operationally defined ligands involved in protein-protein interactions and for optimally identifying an exhaustive set of modular domain-containing proteins implicated in binding with the ligands would be highly desirable.

35           If such a method were available, however, such a method would be useful for the isolation of any polypeptide having a functioning version of any functional domain of

interest. Such a general method would be of tremendous utility in that whole families of related proteins each with its own version of the functional domain of interest could be identified. Knowledge of such related proteins would  
5 contribute greatly to our understanding of various physiological processes, including cell growth or death, malignancy, and immune reactions, to name a few. Such a method would also contribute to the development of increasingly more effective therapeutic, diagnostic, or  
10 prophylactic agents having fewer side effects.

According to the present invention, just such a method is provided.

Regarding SH3 domain-containing proteins, the method of the present invention will contribute greatly to our  
15 understanding of cell growth (Zhu et al., 1993, J. Biol. Chem. 268:1775-1779; Taylor and Shalloway, 1994, Nature 368:867-871), malignancy (Wages et al., 1992, J. Virol. 66:1866-1874; Bruton and Workman, 1993, Cancer Chemother. Pharmacol. 32:1-19), subcellular localization of proteins to the cytoskeleton  
20 and/or cellular membranes (Weng et al., 1993, J. Biol. Chem. 268:14956-14963; Bar-Sagi et al., 1993, Cell 74:83-91), signal transduction (Duchesne et al., 1993, Science 259:525-528), cell morphology (Wages et al., 1992, J. Virol. 66:1866-1874; McGlade et al., 1993, EMBO J. 12:3073-3081), neuronal  
25 differentiation Tanaka et al., 1993, Mol. Cell. Biol. 13:4409-4415), T cell activation (Reynolds et al., 1992, Oncogene 7:1949-1955), and cellular oxidase activity (McAdara and Babior, 1993, Blood 82:A28).

30 Citation of a reference hereinabove shall not be construed as an admission that such is prior art to the present invention.

### 3. SUMMARY OF THE INVENTION

35 In general, the present invention is directed to a method of using isolated, operationally defined ligands involved in binding interactions for optimally identifying an

exhaustive set of compounds binding to such ligands. In one embodiment, the isolated ligands are peptides involved in specific protein-protein interactions and are used to identify a set of novel modular domain-containing proteins that bind to the ligands. Using this method, proteins sharing only modest similarities but a common function can be found.

The present invention is directed to a method of identifying a polypeptide or family of polypeptides having a functional domain of interest. The basic steps of the method comprise: (a) choosing a recognition unit or set of recognition units having a selective affinity for a target molecule with a functional domain of interest; (b) contacting the recognition unit with a plurality of polypeptides; and (c) identifying a polypeptide having a selective binding affinity for the recognition unit, which polypeptide includes the functional domain of interest or a functional equivalent thereof.

In one particular embodiment of the invention, exhaustive screening of proteins having a desired functional domain involves an iterative process by which ligands or recognition units for SH3 domains identified in the first round of screening are used to detect SH3 domain-containing proteins in successive expression library screens.

More particularly, the method of the present invention includes choosing a recognition unit having a selective affinity for a target molecule with a functional domain of interest. With this recognition unit (particularly under the multivalent recognition unit screening conditions taught by the present invention), it has further been discovered that a plurality of polypeptides from various sources can be examined such that certain polypeptides having a selective binding affinity for the recognition unit can be identified. The polypeptides so identified have been shown to include the functional domain of interest; that is, the functional domains found are working versions that are capable of displaying the same binding specificity as the functional domain of interest. Hence, the polypeptides identified by the

present method also possess those attributes of the functional domain of interest which allow these related polypeptides to exhibit the same, similar, or analogous (but functionally equivalent) selective affinity characteristics as the domain of interest of the initial target molecule. By screening the plurality of peptides for recognition unit binding, the methods of the present invention circumvent the limitations of conventional DNA-based screening methods and allow for the identification of highly disparate protein sequences possessing functionally equivalent functional domains.

In specific embodiments of the present invention, the plurality of polypeptides is obtained from the proteins present in a cDNA expression library. The specificity of the polypeptides which bear the functional domain of interest or a functional equivalent thereof for various peptides or recognition units can subsequently be examined, allowing for a greater understanding of the physiological role of particular polypeptide/recognition unit interactions. Indeed, the present invention provides a method of targeted drug discovery based on the observed effects of a given drug candidate on the interaction between a recognition unit-polypeptide pair or a recognition unit and a "panel" of related polypeptides each with a copy or a functional equivalent of (e.g., capable of displaying the same binding specificity and thus binding to the same recognition unit as) the functional domain of interest.

The present invention also provides polypeptides comprising certain amino acid sequences. Moreover, the present invention also provides nucleic acids, including certain DNA constructs comprising certain coding sequences. Using the methods of the present invention, more than eighteen different SH3 domain-containing proteins have been identified, over half of which have not been previously described.

The present inventors have found, unexpectedly, that the valency (i.e., whether it is a monomer, dimer, tetramer, etc.) of the recognition unit that is used to screen an expression library or other source of polypeptides apparently



has a marked effect upon the specificity of the recognition unit-functional domain interaction. The present inventors have discovered that recognition units in the form of small peptides, in multivalent form, have a specificity that is eased but not forfeited. In particular, biotinylated peptides bound to a multivalent (believed to be tetravalent) streptavidin-alkaline phosphatase complex have an unexpected generic specificity. This allows such peptides to be used to screen libraries to identify classes of polypeptides containing functional domains that are similar but not identical in sequence to the peptides' original target functional domains.

The present invention also provides methods for identifying potential new drug candidates (and potential lead compounds) and determining the specificities thereof. For example, knowing that a polypeptide with a functional domain of interest and a recognition unit, e.g., a binding peptide, exhibit a selective affinity for each other, one may attempt to identify a drug that can exert an effect on the polypeptide-recognition unit interaction, e.g., either as an agonist or as an antagonist (inhibitor) of the interaction. With this assay, then, one can screen a collection of candidate "drugs" for the one exhibiting the most desired characteristic, e.g., the most efficacious in disrupting the interaction or in competing with the recognition unit for binding to the polypeptide.

In addition, the present invention also provides certain assay kits and methods of using these assay kits for screening drug candidates for their ability to affect the binding of a polypeptide containing a functional domain to a recognition unit. In a particular aspect of the present invention, the assay kit comprises: (a) a polypeptide containing a functional domain of interest; and (b) a recognition unit having a selective binding affinity for the polypeptide. Yet another assay kit may comprise a plurality of polypeptides, each polypeptide containing a functional domain of interest, in which the functional domain of interest

is a domain selected from the group consisting of an SH1, SH2, SH3, PH, PTB, LIM, armadillo, Notch/ankyrin repeat, zinc finger, leucine zipper, and helix-turn-helix, and at least one recognition unit having a selective affinity for each of the plurality of polypeptides.

Other objects of the present invention will be apparent to those of ordinary skill upon further consideration of the following detailed description.

#### 10 4. DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of the general aspects of a method of identifying recognition units exhibiting a selective affinity for a target molecule with a functional domain of interest. In this illustration, the target molecule is a polypeptide with an SH3 domain, and the recognition units are peptides having a selective affinity for the SH3 domain that are expressed in a phage displayed library.

Figure 2 illustrates the selectivities exhibited by particular recognition units that bind to the Src SH3 domain (in this case, two heptapeptides) for a "panel" of known polypeptides known to contain an SH3 domain. The non-SH3-containing protein, GST, serves as control. RPLPPLP is (SEQ ID NO:45); APPVPPR is (SEQ ID NO:203)

Figure 3 is a schematic representation of the general method of identifying polypeptides with a functional domain of interest by screening a plurality of polypeptides using a suitable recognition unit. In the illustration, the plurality of polypeptides is obtained from a cDNA expression library, and the recognition units are SH3 domain-binding peptides.

Figure 4 illustrates how an SH3 domain-binding peptide can be used to identify other SH3 domain-containing proteins. Shown is a schematic representation of the

progression from initial selection of a target molecule with a functional domain of interest, choice of recognition unit, and identification of polypeptides that have a selective affinity for the recognition unit and include the functional domain of interest or a functional equivalent thereof.

Figure 5 depicts filters from primary (Figure 5B) and tertiary (Figure 5A) screens of a  $\lambda$ cDNA library probed with a biotinylated SH3-binding peptide recognition unit in the form of a complex with streptavidin-alkaline phosphatase (SA-AP). A mouse 16 day embryo cDNA library in  $\lambda$ EXlox was incubated with a multivalent complex formed between biotinylated pSrcCII and SA-AP. The sites of peptide binding were detected by incubation with BCIP (5-bromo-4-chloro-3-indoyl-phosphate-p-toluidine salt) and NBT (nitroblue tetrazolium chloride) for approximately five minutes.

Figure 6 shows an alignment of SH3 domains that illustrates the minimal primary sequence homology among various known SH3 domains. The amino acid sequences shown are SEQ ID NOS:68-111.

Figure 7A is a schematic representation of a population of functional domains represented by the circles. "A" is a recognition unit specific to one circle only. B, on the other hand, recognizes three domains, while B1 and B2 recognize only two each. Figure 7B illustrates an iterative method whereby new recognition units are chosen based on polypeptides uncovered with the first recognition unit(s). These new recognition units lead to the identification of other related polypeptides, etc., expanding the scope of the study to increasingly diverse members of the related population.

Figure 8 illustrates the binding specificity of several SH3 domain recognition units. Biotinylated Class I (pSrcCI) or Class II (pSrcCII) Src SH3 domain recognition

units, Crk SH3 domain recognition units (pCrk), PLC $\gamma$  SH3 domain recognition units (pPLC), and Abl SH3 domain recognition units (pAbl) were tested for binding to the indicated GST-SH3 domain fusion proteins immobilized onto  
5 duplicate microtiter plate wells. Recognition units are listed along the left side of the figure; GST-SH3 domain fusion proteins are listed along the bottom. Recognition units were incubated either as multivalent complexes of biotinylated peptides and streptavidin-horseradish peroxidase  
10 (SA-HRP) (complexed) or as monovalent biotinylated peptides (uncomplexed), followed by incubation with SA-HRP. Average optical densities are shown.

Figure 9 shows a schematic of SH3-domain containing  
15 proteins isolated using the present invention. The name, identity, type of screen, and number of individual clones derived for each sequence are indicated. Diagrams are to scale, with SH3 domains representing approximately 60 amino acids. The abbreviations AR, P, CR, E/P, and SH2 represent  
20 ankyrin repeats, proline-rich segments, Cortactin repeats, glutamate/proline-rich segments, and Src homology 2 domains, respectively. Flared ends represent putative translation initiation sites for individual cDNAs. The Mouse, Human 1, and Human 2 libraries correspond to mouse 16 day embryo, human  
25 bone marrow, and human prostate cancer cDNA libraries, respectively. For a description of the pSrcII and pCort recognition units, see Section 6.1.

Figure 10A and 10B depicts the sequence alignment of  
30 SH3 domains in proteins isolated using the present invention. The name and identity of each clone is indicated. Where appropriate, multiple SH3 domains from the same polypeptide are designated A, B, C, etc., from N- to C-terminal. Periods indicate gaps introduced to maximize alignment of similar  
35 residues. Positions corresponding to conserved residues shown to be involved in ligand binding in the SH3 domains of Src and Grb2/Sem5 (Tomasetto et al., 1995, Genomics 28:367-376) are

presented in bold and underlined, respectively. Primary structures of SH3P1-8 and SH3P10-13 correspond to mouse, SH3P15-18, clone 5, 34, 40, 41, 45, 53, 55, 56, and 65 to human, and SH3P9 and SH3P14 to mouse (m) or human (h) cDNA clones. For sequence comparison, the sequence of the mouse c-Src SH3 domain (GenBank accession number P41240) is shown. The GenBank accession numbers for mouse Cortactin, SPY75/HS1, Crk, and human MLN50, Lyn, Fyn, and Src are U03184, D42120, S72408, X82456, M16038, P06241, and P41240, respectively. The amino acid sequences shown are SEQ ID NOs:112-140.

Figure 11 depicts the specificity continuum described in Section 5.2.1. "SA-AP peptide complex" represents the multivalent (believed to be tetravalent) complex of streptavidin-alkaline phosphatase and biotinylated peptide described in that section.

Figure 12 depicts the results of experiments in which peptide recognition units were synthesized and tested for their ability to bind to novel SH3 domains described in Sections 6.1 and 6.1.1. A minus indicates no binding; a plus indicates binding, with the number of pluses indicating the strength of binding. For further details, see Section 6.2. The amino acid sequences shown are SEQ ID NOs:141-168.

Figure 13 depicts more data from the experiment depicted in Figure 12. The amino acid sequences shown are SEQ ID NOs:169-188.

Figure 14 illustrates the effect of preconjugation with streptavidin-alkaline phosphatase on the affinity of biotinylated peptides for SH3 domains. See Section 6.3.1 for details.

Figure 15 illustrates the effect of preconjugation with streptavidin-alkaline phosphatase on the specificity of biotinylated peptides for GST-SH3 domain fusion proteins that

have been immobilized on nylon membranes. See Section 6.3.2 for details.

Figure 16 illustrates the effect of preconjugation with streptavidin-alkaline phosphatase on the specificity of biotinylated peptides for proteins containing SH3 domains expressed by cDNA clones. See Section 6.3.3 for details.

Figure 17 illustrates a strategy for exhaustively screening an expression library for SH3 domain-containing proteins. A peptide recognition unit is generated by screening a combinatorial peptide library for binders to an SH3 domain expressed bacterially as a GST fusion protein. This peptide is then used as a multivalent streptavidin-biotinylated peptide complex to screen for a subset of the SH3 domain-containing proteins represented in a cDNA expression library. A combinatorial library is once again used to identify recognition units of SH3 domains identified in the first expression library screen; these recognition units identify overlapping sets of proteins from the expression library. With multiple iterations of this process, it should be possible to clone systematically all SH3 domains represented in a given cDNA expression library.

Figure 18 depicts the nucleotide sequence of SH3P1, mouse p53bp2 (SEQ ID NO:5).

Figure 19 depicts the amino acid sequence of SH3P1, mouse p53bp2 (SEQ ID NO:6).

Figure 20 depicts the nucleotide sequence of SH3P2, a novel mouse gene (SEQ ID NO:7).

Figure 21 depicts the amino acid sequence of SH3P2, a novel mouse gene (SEQ ID NO:8).

Figure 22 depicts the nucleotide sequence of SH3P3,  
a novel mouse gene (SEQ ID NO:9).

Figure 23 depicts the amino acid sequence of SH3P3,  
5 a novel mouse gene (SEQ ID NO:10).

Figure 24 depicts the nucleotide sequence of SH3P4,  
a novel mouse gene (SEQ ID NO:11).

10 Figure 25 depicts the amino acid sequence of SH3P4,  
a novel mouse gene (SEQ ID NO:12).

Figure 26 depicts the nucleotide sequence of SH3P5,  
mouse Cortactin (SEQ ID NO:13).

15 Figure 27 depicts the amino acid sequence of SH3P5,  
mouse Cortactin (SEQ ID NO:14).

Figure 28 depicts the nucleotide sequence of SH3P6,  
20 mouse MLN50 (SEQ ID NO:15).

Figure 29 depicts the amino acid sequence of SH3P6,  
mouse MLN50 (SEQ ID NO:16).

25 Figure 30 depicts the nucleotide sequence of SH3P7,  
a novel mouse gene (SEQ ID NO:17).

Figure 31 depicts the amino acid sequence of SH3P7,  
a novel mouse gene (SEQ ID NO:18).

30 Figure 32 depicts the nucleotide sequence of SH3P8,  
a novel mouse gene (SEQ ID NO:19).

Figure 33 depicts the amino acid sequence of SH3P8,  
35 a novel mouse gene (SEQ ID NO:20)..

Figure 34 depicts the nucleotide sequence of SH3P9, a novel mouse gene (SEQ ID NO:21).

Figure 35 depicts the amino acid sequence of SH3P9, a novel mouse gene (SEQ ID NO:22).

Figure 36 depicts the nucleotide sequence of SH3P9, a novel human gene (SEQ ID NO:23).

10 Figure 37 depicts the amino acid sequence of SH3P9, a novel human gene (SEQ ID NO:24).

Figure 38 depicts the nucleotide sequence of SH3P10, mouse HS1 (SEQ ID NO:25).

15

Figure 39 depicts the amino acid sequence of SH3P10, mouse HS1 (SEQ ID NO:26).

Figure 40 depicts the nucleotide sequence of SH3P11, 20 mouse Crk (SEQ ID NO:27).

Figure 41 depicts the amino acid sequence of SH3P11, mouse Crk (SEQ ID NO:28).

25 Figure 42A depicts the nucleotide sequence from positions 1-2600 of SH3P12, a novel mouse gene (a portion of SEQ ID NO:29).

Figure 42B depicts the nucleotide sequence from 30 positions 2601-3335 of SH3P12, a novel mouse gene (a portion of SEQ ID NO:29).

Figure 43 depicts the amino acid sequence of SH3P12, a novel mouse gene (SEQ ID NO:30).

35

Figure 44 depicts the nucleotide sequence of SH3P13, a novel mouse gene (SEQ ID NO:31).



Figure 45 depicts the amino acid sequence of SH3P13, a novel mouse gene (SEQ ID NO:32).

Figure 46A depicts the nucleotide sequence from 5 positions 1-2400 of SH3P14, mouse H74 (a portion of SEQ ID NO:33).

Figure 46B depicts the nucleotide sequence from positions 2351-4091 of SH3P14, mouse H74 (a portion of SEQ ID NO:33).

Figure 47 depicts the amino acid sequence of SH3P14, mouse H74 (SEQ ID NO:34).

Figure 48 depicts the nucleotide sequence of SH3P14, human H74 (SEQ ID NO:35).

Figure 49 depicts the amino acid sequence of SH3P14, human H74 (SEQ ID NO:36).

Figure 50 depicts the nucleotide sequence of SH3P17, a novel human gene (SEQ ID NO:37).

Figure 51 depicts the amino acid sequence of SH3P17, a novel human gene (SEQ ID NO:38).

Figure 52A depicts the nucleotide sequence of SH3P18, a novel human gene (SEQ ID NO:39).

Figure 53 depicts the amino acid sequence of SH3P18, a novel human gene (SEQ ID NO:40).

Figure 54 depicts the nucleotide sequence of clone 55, a novel human gene (SEQ ID NO:189).

Figure 55 depicts the amino acid sequence of clone 55, a novel human gene (SEQ ID NO:190).

Figure 56 depicts the nucleotide sequence of clone 56, a novel human gene (SEQ ID NO:191).

Figure 57 depicts the amino acid sequence of clone 56, a novel human gene (SEQ ID NO:192).

Figure 58A depicts the nucleotide sequence from position 1-1720 of clone 65, a novel human gene (a portion of SEQ ID NO:193).

10

Figure 58B depicts the nucleotide sequence from position 1721-2873 of clone 65, a novel human gene (a portion of SEQ ID NO:193).

15 Figure 59 depicts the amino acid sequence of clone 65, a novel human gene (SEQ ID NO:194).

Figure 60 depicts the nucleotide sequence of clone 34, a novel human gene (SEQ ID NO:195).

20

Figure 61A depicts a portion of the amino acid sequence of clone 34, a novel human gene (a portion of SEQ ID NO:196).

25 Figure 61B depicts a portion of the amino acid sequence of clone 34, a novel human gene (a portion of SEQ ID NO:196).

Figure 62 depicts the nucleotide sequence of clone 41, a novel human gene (SEQ ID NO:197).

30

Figure 63A depicts a portion of the amino acid sequence of clone 41, a novel human gene (a portion of SEQ ID NO:198).

35

Figure 63B depicts a portion of the amino acid sequence of clone 41, a novel human gene (a portion of SEQ ID NO:198).

5           Figure 64A depicts the nucleotide sequence of clone 53, a novel human gene (SEQ ID NO:199).

Figure 65A depicts a portion of the amino acid sequence of clone 53, a novel human gene (a portion of SEQ ID  
10 NO:200).

Figure 65B depicts a portion of the amino acid sequence of clone 53, a novel human gene (a portion of SEQ ID NO:200).

15

Figure 66A and 66B depicts the nucleotide sequence (SEQ ID NO:220) and amino acid sequence (SEQ ID NO:221) of clone 5, a novel human gene.

## 20 5.    DETAILED DESCRIPTION OF THE INVENTION

As stated above, the present invention is related broadly to certain polypeptides having a functional domain of interest and is directed to methods of identifying and using these polypeptides. The present invention is also directed to  
25 a method of using isolated, operationally defined ligands involved in binding interactions for optimally identifying an exhaustive set of compounds binding such ligands and to compounds, target molecules, and, in one embodiment, polypeptides having a functional domain of interest and to  
30 methods of using these compounds. The detailed description that follows is provided to elucidate the invention further and to assist further those of ordinary skill who may be interested in practicing particular aspects of the invention.

First, certain definitions are in order.

35 Accordingly, the term "polypeptide" refers to a molecule comprised of amino acid residues joined by peptide (i.e., amide) bonds and includes proteins and peptides. Hence, the

polypeptides of the present invention may have single or multiple chains of covalently linked amino acids and may further contain intrachain or interchain linkages comprised of disulfide bonds. Some polypeptides may also form a subunit of  
5 a multiunit macromolecular complex. Naturally, the polypeptides can be expected to possess conformational preferences and to exhibit a three-dimensional structure. Both the conformational preferences and the three-dimensional structure will usually be defined by the polypeptide's primary  
10 (i.e., amino acid) sequence and/or the presence (or absence) of disulfide bonds or other covalent or non-covalent intrachain or interchain interactions.

The polypeptides of the present invention can be any size. As can be expected, the polypeptides can exhibit a wide  
15 variety of molecular weights, some exceeding 150 to 200 kilodaltons (kD). Typically, the polypeptides may have a molecular weight ranging from about 5,000 to about 100,000 daltons. Still others may fall in a narrower range, for example, about 10,000 to about 75,000 daltons, or about 20,000  
20 to about 50,000 daltons.

The phrase "functional domain" refers to a region of a polypeptide which affords the capacity to perform a particular function of interest. This function may give rise to a biological, chemical, or physiological consequence that  
25 may be reversible or irreversible and which may include, but not be limited to, protein-protein interactions (e.g., binding interactions) involving the functional domain, a change in the conformation or a transformation into a different chemical state of the functional domain or of molecules acted upon by  
30 the functional domain, the transduction of an intracellular or intercellular signal, the regulation of gene or protein expression, the regulation of cell growth or death, or the activation or inhibition of an immune response. Furthermore, the functional domain of interest is defined by a particular  
35 functional domain that is present in a given target molecule. A discussion of the selection of a particular functional domain-containing target molecule is presented further below.

Many functional domains tend to be modular in that such domains may occur one or more times in a given polypeptide (or target molecule) or may be found in a family of different polypeptides. When found more than once in a  
5 given polypeptide or in different polypeptides, the modular functional domain may possess substantially the same structure, in terms of primary sequence and/or three-dimensional space, or may contain slight or great variations or modifications among the different versions of the  
10 functional domain of interest.

What is important, however, is that these related functional domains retain the functional aspects of the functional domain of interest present in the target molecule. It is stressed that, indeed, it is this functional  
15 relationship among two or more possible versions of a functional domain of interest which may be identified, defined, and exploited by the methods of the present invention. In a preferred aspect, the function of interest is the ability to bind to a molecule (e.g., a peptide) of  
20 interest.

The present invention provides a general strategy by which recognition units that bind to a functional domain-containing molecule can be used to screen expression libraries of genes (e.g., cDNA, genomic libraries) systematically for  
25 novel functional domain-containing proteins. In specific embodiments, the recognition units are prior isolated from a random peptide library, or are known peptide ligands or recognition units, or are recognition units that are identified by database searches for sequences having homology  
30 to a peptide recognition unit having the binding specificity of interest. Using the methods of the present invention, it is possible to exhaustively screen an expression library for proteins with a given functional domain.

In the prior art, novel genes (and thus their  
35 encoded protein products) are most commonly identified from cDNA libraries. Generally, an appropriate cDNA library is screened with a probe that is either an oligonucleotide or an

antibody. In either case, the probe must be specific enough for the gene that is to be identified to pick that gene out from a vast background of non-relevant genes in the library. It is this need for a specific probe that is the highest  
5 hurdle that must be overcome in the prior art identification of novel genes. Another method of identifying genes from cDNA libraries is through use of the polymerase chain reaction (PCR) to amplify a segment of a desired gene from the library. PCR requires that oligonucleotides having sequence similarity  
10 to the desired gene be available.

If the probe used in prior art methods is a nucleic acid, the cDNA library may be screened without the need for expressing any protein products that might be encoded by the cDNA clones. If the probe used in prior art methods is an  
15 antibody, then it is necessary to build the cDNA library into a suitable expression vector. For a comprehensive discussion of the art of identifying genes from cDNA libraries, see Sambrook, Fritsch, and Maniatis, "Construction and Analysis of cDNA Libraries," Chapter 8 in Cloning, A Laboratory Manual, 2d  
20 ed., Cold Spring Harbor Laboratory Press, 1989. See also Sambrook, Fritsch, and Maniatis, "Screening Expression Libraries with Antibodies and Oligonucleotides," Chapter 12 in Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, 1989.

25 As an alternative to cDNA libraries, genomic libraries are used. When genomic libraries are used in prior art methods, the probe is virtually always a nucleic acid probe. See Sambrook, Fritsch, and Maniatis, "Analysis and Cloning of Eukaryotic Genomic DNA," Chapter 9 in Cloning, A  
30 Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, 1989.

In the prior art, nucleic acid probes used in screening libraries are often based upon the sequence of a known gene that is thought to be homologous to a gene that it  
35 is desired to isolate. The success of the procedure depends upon the degree of homology between the probe and the target gene being sufficiently high. Probes based upon the sequences

of known functional domains in proteins had limited value because, while the sequences of the functional domains were similar enough to allow for their recognition as shared domains, the similarity was not so high that the probes could  
5 be used to screen cDNA or genomic libraries for genes containing the functional domains.

PCR may also be used to identify genes from genomic libraries. However, as in the case of using PCR to identify genes from cDNA libraries, this requires that oligonucleotides  
10 having sequence similarity to the desired gene be available.

Using the screening methods provided by the present invention, DNA encoding proteins having a desired functional domain that would not be readily identified by sequence homology can be identified by functional binding specificity  
15 to recognition units. By virtue of an ease in specificity of binding requirements conferred by the screening methods of the present invention, many novel, functionally homologous, functional domain-containing proteins can be identified. Although not intending to be bound by any mechanistic  
20 explanation, this ease in binding specificity is believed to be the result of the use of a multivalent peptide recognition unit used to screen the gene library, preferably of a valency greater than bivalent, more preferably tetravalent or greater, and most preferably the streptavidin-biotinylated peptide  
25 recognition unit complex.

In one particular embodiment of the invention, exhaustive screening of proteins having a desired functional domain involves an iterative process by which recognition units for SH3 domains identified in the first round of  
30 screening are used to detect SH3 domain-containing proteins in successive expression library screens (see Figure 17). This strategy enables one to search "sequence space" in what might be thought of as ever-widening circles with each successive cycle. This iterative strategy can be initiated even when  
35 only one functional domain-containing protein and recognition unit are available.

This iterative process is not limited to proteins containing SH3 domains. Members within a class of other functional domains also tend to have overlapping, or at least similar recognition unit preferences, are structurally stable, and often confer similar binding properties to a wide variety of proteins. These characteristics predict that the methods of the present invention will be applicable to a wide variety of functional domain-containing proteins in addition to their applicability to SH3 domain-containing proteins.

#### 5.1. Discovery of Novel Genes and Polypeptides Containing Functional Domains

The present invention provides methods for the identification of one or more polypeptides (in particular, a "family" of polypeptides, including the target molecule) that contains a functional domain of interest that either corresponds to or is the functional equivalent of a functional domain of interest present in a predetermined target molecule.

The present invention provides a mechanism for the rapid identification of genes (e.g., cDNAs) encoding virtually any functional domain of interest. By screening cDNA libraries or other sources of polypeptides for recognition unit binding rather than sequence similarity, the present invention circumvents the limitations of conventional DNA-based screening methods and allows for the identification of highly disparate protein sequences possessing equivalent functional activities. The ability to isolate entire repertoires of proteins containing particular modular functional domains will prove invaluable both in molecular biological investigations of the genome and in bringing new targets into drug discovery programs.

It should likewise be apparent that a wide range of polypeptides having a functional domain of interest can be identified by the process of the invention, which process comprises:

(a) contacting a multivalent recognition unit complex with a plurality of polypeptides; and



(b) identifying a polypeptide having a selective binding affinity for said recognition unit complex.

In a specific embodiment, the process comprises:

(a) contacting a multivalent recognition unit  
5 complex with a plurality of polypeptides from which it is desired to identify a polypeptide having selective binding affinity for the recognition unit, in which the valency of the recognition unit in the complex is at least two, or at least four; and

10 (b) identifying, and preferably recovering, a polypeptide having a selective binding affinity for the recognition unit complex.

In another specific embodiment, the process comprises a method of identifying at least one polypeptide  
15 comprising a functional domain of interest, said method comprising:

(a) contacting one or more multivalent recognition unit complexes with a plurality of polypeptides; and

(b) identifying at least one polypeptide having  
20 selective binding affinity for at least one of said recognition unit complexes.

In another specific embodiment, the process comprises:

(a) contacting a multivalent recognition unit  
25 complex, which complex comprises (i) avidin or streptavidin, and (ii) biotinylated recognition units, with a plurality of polypeptides from a cDNA expression library, in which the recognition unit is a peptide having in the range of 6 to 60 amino acid residues; and

30 (b) identifying a polypeptide having a selective binding affinity for said recognition unit complex.

In another specific embodiment, the process comprises a method of identifying a polypeptide having an SH3 domain of interest comprising:

35 (a) contacting a multivalent recognition unit complex, which complex comprises (i) avidin or streptavidin, and (ii) biotinylated recognition units, with a plurality of

polypeptides from a cDNA expression library, in which the recognition unit is a peptide having in the range of 6 to 60 amino acid residues and which selectively binds an SH3 domain; and

- 5 (b) identifying a polypeptide having a selective binding affinity for said recognition unit complex.

In another specific embodiment, the process comprises a method of identifying a polypeptide having a functional domain of interest or a functional equivalent  
10 thereof comprising:

(a) screening a random peptide library to identify a peptide that selectively binds a functional domain of interest; and

(b) screening a cDNA or genomic expression library  
15 with said peptide or a binding portion thereof to identify a polypeptide that selectively binds said peptide.

In a specific embodiment of the above method, the screening step (b) is carried out by use of said peptide in the form of multiple antigen peptides (MAP) or by use of said  
20 peptide cross-linked to bovine serum albumin or keyhole limpet hemocyanin.

In another specific embodiment, the process comprises a method of identifying a polypeptide having a functional domain of interest or a functional equivalent  
25 thereof comprising:

(a) screening a random peptide library to identify a plurality of peptides that selectively bind a functional domain of interest;

(b) determining at least part of the amino acid  
30 sequences of said peptides;

(c) determining a consensus sequence based upon the determined amino acid sequences of said peptides; and

(d) screening a cDNA or genomic expression library with a peptide comprising the consensus sequence to identify a  
35 polypeptide that selectively binds said peptide.

In another specific embodiment, the process comprises a method of identifying a polypeptide having a

functional domain of interest or a functional equivalent thereof comprising:

(a) screening a random peptide library to identify a first peptide that selectively binds a functional domain of  
5 interest;

(b) determining at least part of the amino acid sequence of said first peptide;

(c) searching a database containing the amino acid sequences of a plurality of expressed natural proteins to  
10 identify a protein containing an amino acid sequence homologous to the amino acid sequence of said first peptide;  
and

(d) screening a cDNA or genomic expression library with a second peptide comprising the sequence of said protein  
15 that is homologous to the amino acid sequence of said first peptide.

The identified polypeptide identified by the above-described methods thus should contain the functional domain of interest or a functional equivalent thereof (that is, having a  
20 functional domain that is identical, or having a functional domain that differs in sequence but is capable of binding to the same recognition unit). In a particular embodiment, the polypeptide identified is a novel polypeptide. In a preferred embodiment, the recognition unit that is used to form the  
25 multivalent recognition unit complex is isolated or identified from a random peptide library.

In a specific embodiment, the present invention provides amino acid sequences and DNA sequences encoding novel proteins containing SH3 domains. The SH3 domains vary in  
30 sequence but retain binding specificity to an SH3 domain recognition unit. Also provided are fragments and derivatives of the novel proteins containing SH3 domains as well as DNA sequences encoding the same. It will be apparent to one of ordinary skill in the art that also provided are proteins that  
35 vary slightly in sequence from the novel proteins by virtue of conservative amino acid substitutions. It will also be apparent to one of ordinary skill in the art that the novel

proteins may be expressed recombinantly by standard methods. The novel proteins may also be expressed as fusion proteins with a variety of other proteins, e.g., glutathione S-transferase.

5           The present invention provides a purified polypeptide comprising an SH3 domain, said SH3 domain having an amino acid sequence selected from the group consisting of: SEQ ID NOS: 113-115, 118-121, 125-128, 133-139, 204-218, and 219. Also provided is a purified DNA encoding the  
10 polypeptide.

          Also provided is a purified polypeptide comprising an SH3 domain, said polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOS: 8, 10, 12, 18, 20, 22, 24, 30, 32, 38, 40, 190, 192, 194, 196, 198, 200,  
15 and 221. Also provided is a purified DNA encoding the polypeptide.

          Also provided is a purified DNA encoding an SH3 domain, said DNA having a sequence selected from the group consisting of SEQ ID NOS: 7, 9, 11, 17, 19, 21, 23, 29, 31,  
20 37, 39, 189, 191, 193, 195, 197, 199, and 220. Also provided is a nucleic acid vector comprising this purified DNA. Also provided is a recombinant cell containing this nucleic acid vector.

          Also provided is a purified DNA encoding a  
25 polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NOS: 8, 10, 12, 18, 20, 22, 24, 30, 32, 38, 40, 190, 192, 194, 196, 198, 200, and 221. Also provided is a nucleic acid vector comprising this purified DNA. Also provided is a recombinant cell containing this  
30 nucleic acid vector.

          Also provided is a purified DNA encoding a polypeptide comprising an amino acid sequence selected from the group consisting of: SEQ ID NOS: 113-115, 118-121, 125-128, 133-139, 204-218, and 219. Also provided is a nucleic acid  
35 vector comprising this purified DNA. Also provided is a recombinant cell containing this nucleic acid vector.

Also provided is a purified molecule comprising an SH3 domain of a polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NO: 8, 10, 12, 18, 20, 22, 24, 30, 32, 38, 40, 190, 192, 194, 196, 198, 200, 5 and 221.

Also provided is a fusion protein comprising (a) an amino acid sequence comprising an SH3 domain of a polypeptide having the amino acid sequence of SEQ ID NO: 8, 10, 12, 18, 20, 22, 24, 30, 32, 38, 40, 190, 192, 194, 196, 198, 200, and 10 221 joined via a peptide bond to (b) an amino acid sequence of at least six, or ten, or twenty amino acids from a different polypeptide. Also provided is a purified DNA encoding the fusion protein. Also provided is a nucleic acid vector comprising the purified DNA encoding the fusion protein. Also 15 provided is a recombinant cell containing this nucleic acid vector. Also provided is a method of producing this fusion protein comprising culturing a recombinant cell containing a nucleic acid vector encoding said fusion protein such that said fusion protein is expressed, and recovering the expressed 20 fusion protein.

The present invention also provides a purified nucleic acid hybridizable to a nucleic acid having a sequence selected from the group consisting of: SEQ ID NOs: 7, 9, 11, 17, 19, 21, 23, 29, 31, 37, 39, 189, 191, 193, 195, 197, 199, 25 and 220.

The present invention also provides antibodies to a polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NOs: 113-115, 118-121, 125-128, 133-139, 204-218, and 219.

30 The present invention also provides antibodies to a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 10, 12, 18, 20, 22, 24, 30, 32, 38, 40, 190, 192, 194, 196, 198, 200, and 221.

It is demonstrated by way of example herein that 35 recognition units that comprise SH3 domain ligands derived from combinatorial peptide libraries may be used in the methods of the present invention as probes for the rapid

discovery of novel proteins containing SH3 functional domains. The methods of the present invention require no prior knowledge of the characteristics of a SH3 domain's natural cellular ligand to initiate the process of discovery. One  
5 needs only enough purified SH3 domain-containing protein (by way of example, 1-5 $\mu$ g) to select peptides from a random peptide library. In addition, because the methods of the present invention identify novel proteins from cDNA expression libraries based only on their binding properties, low primary  
10 sequence identity between the target SH3 domain and the SH3 domains of the novel proteins discovered need not be a limitation, provided some functional similarity between these SH3 domains is conserved. Also, the methods of the present invention are rapid, require inexpensive reagents, and employ  
15 simple and well established laboratory techniques.

Using these methods, more than eighteen different SH3 domain-containing proteins have been identified, over half of which have not been previously described. While certain of these previously unknown proteins are clearly related to known  
20 genes such as amphiphysin and drebrin, others constitute new classes of signal transduction and/or cytoskeletal proteins. These include SH3P17 and SH3P18, two members of a new family of adaptor-like proteins comprised of multiple SH3 domains; SH3P12, a novel protein with three SH3 domains and a region  
25 similar to the extracellular peptide hormone sorbin; and SH3P4, SH3P8, and SH3P13, three members of a third new family of SH3-containing proteins. These novel proteins are described more fully in Sections 6.1 and 6.1.1. The high  
30 incidence of novel proteins identified by the methods of the present invention indicates that a large number of SH3 domain-containing proteins remain to be discovered by application of the methods of the invention.

One of ordinary skill in the art would recognize that the above-described novel proteins need not be used in  
35 their entirety in the various applications of those proteins described herein. In many cases it will be sufficient to employ that portion of the novel protein that contains the

functional (e.g., SH3) domain. Such exemplary portions of SH3 domain-containing proteins are shown in Figure 10A and 10B. Accordingly, the present invention provides derivatives (e.g., fragments and molecules comprising these fragments) of novel  
5 proteins that contain SH3 domains, e.g., as shown in Figure 10A and 10B. Nucleic acids encoding these fragments or other derivatives are also provided.

In another embodiment, the present invention includes a method of identifying one or more novel  
10 polypeptides having an SH3 domain, said method comprising:  
    (a) identifying a recognition unit having a selective affinity for the SH3 domain by screening a peptide library with the SH3 domain;  
    (b) producing said recognition unit;  
15      (c) contacting said recognition unit with a source of polypeptides; and  
    (d) identifying one or more novel polypeptides having a selective affinity for said recognition unit, which polypeptides comprise the SH3 domain.

20

#### 5.1.1 Functional Domains

Functional domains of interest in the practice of the present invention can take many forms and may perform a variety of functions. For example, such functional domains  
25 may be involved in a number of cellular, biochemical, or physiological processes, such as cellular signal transduction, transcriptional regulation, translational regulation, cell adhesion, migration or transport, cytokine secretion and other aspects of the immune response, and the like. In particular  
30 embodiments of the present invention, the functional domains of interest may consist of regions known as SH1, SH2, SH3, PH, PTB, LIM, armadillo, and Notch/ankyrin repeat. See, e.g., Pawson, 1995, Nature 373:573-580; Cohen et al., 1995, Cell 80:237-248. Functional domains may also be chosen from among  
35 regions known as zinc fingers, leucine zippers, and helix-turn-helix or helix-loop-helix. Certain functional domains may be binding domains, such as DNA-binding domains or actin-

binding domains. Still other functional domains may serve as sites of catalytic activity.

In one embodiment of the invention, a suitable target molecule containing the chosen functional domain of interest is selected. In the case of an SH3 domain, for example, a number of proteins (or functional domain-containing derivatives or analogs thereof) may be selected as the target molecule, including but not limited to, the Src family of proteins: Fyn, Lck, Lyn, Src, or Yes. Still other proteins contain an SH3 domain and can be used, including, but not limited to: Abl, Crk, Nck (other oncogenes), Grb2, PLC $\gamma$ , RasGAP (proteins involved in signal transduction), ABP-1, myosin-1, spectrin (proteins found in the cytoskeleton), and neutrophil NADPH oxidase (an enzyme). In the case of a catalytic site, any catalytically active protein, such as an enzyme, can be used, particularly one whose catalytic site is known. For example, the catalytic site of the protein glutathione S-transferase (GST) can be used. Other target molecules that possess catalytic activity may include, but are not limited to, protein serine/threonine kinases, protein tyrosine kinases, serine proteases, DNA or RNA polymerases, phospholipases, GTPases, ATPases, PI-kinases, DNA methylases, metabolic enzymes, or protein glycosylases.

#### 25        5.1.2.    Recognition Units

By the phrase "recognition unit," is meant any molecule having a selective affinity for the functional domain of the target molecule and, preferably, having a molecular weight of up to about 20,000 daltons. In a particular embodiment of the invention, the recognition unit has a molecular weight that ranges from about 100 to about 10,000 daltons.

Accordingly, preferred recognition units of the present invention possess a molecular weight of about 100 to about 5,000 daltons, preferably from about 100 to about 2,000 daltons, and most preferably from about 500 to about 1,500 daltons. As described further below, the recognition unit of



the present invention can be a peptide, a carbohydrate, a nucleoside, an oligonucleotide, any small synthetic molecule, or a natural product. When the recognition unit is a peptide, the peptide preferably contains about 6 to about 60 amino acid  
5 residues.

When the recognition unit is a peptide, the peptide can have less than about 140 amino acid residues; preferably, the peptide has less than about 100 amino acid residues; preferably, the peptide has less than about 70 amino acid  
10 residues; preferably, the peptide has 20 to 50 amino acid residues; most preferably, the peptide has about 6 to 60 amino acid residues.

The peptide recognition units are preferably in the form of a multivalent peptide complex comprising avidin or  
15 streptavidin (optionally conjugated to a label such as alkaline phosphatase or horseradish peroxidase) and biotinylated peptides.

According to the present invention, a recognition unit (preferably in the form of a multivalent recognition unit  
20 complex) is used to screen a plurality of expression products of gene sequences containing nucleic acid sequences that are present in native RNA or DNA (e.g., cDNA library, genomic library).

The step of choosing a recognition unit can be  
25 accomplished in a number of ways that are known to those of ordinary skill, including but not limited to screening cDNA libraries or random peptide libraries for a peptide that binds to the functional domain of interest. See, e.g., Yu et al., 1994, Cell 76, 933-945; Sparks et al., 1994, J. Biol. Chem.  
30 269, 23853-23856. Alternatively, a peptide or other small molecule or drug may be known to those of ordinary skill to bind to a certain target molecule and can be used. The recognition unit can even be synthesized from a lead compound, which again may be a peptide, carbohydrate, oligonucleotide,  
35 small drug molecule, or the like. The recognition unit can also be identified for use by doing searches (preferably via database) for molecules having homology for other, known

recognition unit(s) having the ability to selectively bind to the functional domain of interest.

In a specific embodiment, the step of selecting a recognition unit for use can be effected by, e.g., the use of  
5 diversity libraries, such as random or combinatorial peptide or nonpeptide libraries, which can be screened for molecules that specifically bind to the functional domain of interest, e.g., an SH3 domain. Many libraries are known in the art that can be used, e.g., chemically synthesized libraries,  
10 recombinant (e.g., phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor et al., 1991, Science 251:767-773; Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991,  
15 Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412;  
20 Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in  
25 Scott and Smith, 1990, Science 249:386-390; Devlin et al., 1990, Science, 249:404-406; Christian, R.B., et al., 1992, J. Mol. Biol. 227:711-718); Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay et al., 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

30 *In vitro* translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis et al., 1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

By way of examples of nonpeptide libraries, a  
35 benzodiazepine library (see e.g., Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci.

USA 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

The variety of non-peptide libraries that are useful in the present invention is great. For example, Ecker and Crooke, 1995, Bio/Technology 13:351-360 list benzodiazapines, hydantoins, piperazinediones, biphenyls, sugar analogs,  $\beta$ -mercaptoketones, arylacetic acids, acylpiperidines, benzopyrans, cubanes, xanthines, aminimides, and oxazolones as among the chemical species that form the basis of various libraries.

Non-peptide libraries can be classified broadly into two types: decorated monomers and oligomers. Decorated monomer libraries employ a relatively simple scaffold structure upon which a variety of functional groups is added. Often the scaffold will be a molecule with a known useful pharmacological activity. For example, the scaffold might be the benzodiazapine structure.

Non-peptide oligomer libraries utilize a large number of monomers that are assembled together in a ways that create new shapes that depend on the order of the monomers. Among the monomer units that have been used are carbamates, pyrrolinones, and morpholinos. Peptoids, peptide-like oligomers in which the side chain is attached to the  $\alpha$  amino group rather than the  $\alpha$  carbon, form the basis of another version of non-peptide oligomer libraries. The first non-peptide oligomer libraries utilized a single type of monomer and thus contained a repeating backbone. Recent libraries have utilized more than one monomer, giving the libraries added flexibility.

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, e.g., the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390;

Fowlkes et al., 1992; BioTechniques 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu et al., 1994, Cell 76:933-945; Staudt et al., 1988, Science 241:577-580; Bock et al., 1992, Nature 355:564-566; Tuerk et al., 5 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

10 In a specific embodiment, screening to identify a recognition unit can be carried out by contacting the library members with an SH3 domain immobilized on a solid phase and harvesting those library members that bind to the SH3 domain. Examples of such screening methods, termed "panning"

15 techniques are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment, the two-hybrid system for 20 selecting interacting proteins in yeast (Fields and Song, 1989, Nature 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify recognition units that specifically bind to SH3 domains.

Where the recognition unit is a peptide, the peptide 25 can be conveniently selected from any peptide library, including random peptide libraries, combinatorial peptide libraries, or biased peptide libraries. The term "biased" is used herein to mean that the method of generating the library is manipulated so as to restrict one or more parameters that 30 govern the diversity of the resulting collection of molecules, in this case peptides.

Thus, a truly random peptide library would generate a collection of peptides in which the probability of finding a particular amino acid at a given position of the peptide is 35 the same for all 20 amino acids. A bias can be introduced into the library, however, by specifying, for example, that a lysine occur every fifth amino acid or that positions 4, 8,

and 9 of a decapeptide library be fixed to include only arginine. Clearly, many types of biases can be contemplated, and the present invention is not restricted to any particular bias. Furthermore, the present invention contemplates  
5 specific types of peptide libraries, such as phage-displayed peptide libraries and those that utilize a DNA construct comprising a lambda phage vector with a DNA insert.

As mentioned above, in the case of a recognition unit that is a peptide, the peptide may have about 6 to less  
10 than about 60 amino acid residues, preferably about 6 to about 25 amino acid residues, and most preferably, about 6 to about 15 amino acids. In another embodiment, a peptide recognition unit has in the range of 20-100 amino acids, or 20-50 amino acids. In the case of a bile acid receptor, for example, the  
15 recognition unit may be a bile acid, such as cholic acid or cholesterol, and may have a molecular weight of about 300 to about 600. If the functional domain relates to transcriptional control, the recognition unit may be a portion of a transcriptional factor, which may bind to a region of a  
20 gene of interest or to an RNA polymerase. The recognition unit may even be a nucleoside analog, such as cordycepin or the triphosphate thereof, capable of inhibiting RNA biosynthesis. The recognition unit may also be the carbohydrate portion of a glycoprotein, which may have a  
25 selective affinity for the asialoglycoprotein receptor, or the repeating glucan unit that exhibits a selective affinity for a cellulose binding domain or the active site of heparinase.

The selected recognition unit can be obtained by chemical synthesis or recombinant expression. It is  
30 preferably purified prior to use in screening a plurality of gene sequences.

#### 5.1.3. Screening a Source of Polypeptides

After the recognition unit is chosen for use, the  
35 recognition unit is then contacted with a plurality of polypeptides, preferably containing a functional domain. In a particular embodiment of the invention, the plurality of

polypeptides is obtained from a polypeptide expression library. The polypeptide expression library may be obtained, in turn, from cDNA, fragmented genomic DNA, and the like. In a specific embodiment, the library that is screened is a cDNA library of total poly A+ RNA of an organism, in general, or of a particular cell or tissue type or developmental stage or disease condition or stage. The expression library may utilize a number of expression vehicles known to those of ordinary skill, including but not limited to, recombinant bacteriophage, lambda phage, M13, a recombinant plasmid or cosmid, and the like.

The plurality of polypeptides or the DNA sequences encoding same may be obtained from a variety of natural or unnatural sources, such as a procaryotic or a eucaryotic cell, either a wild type, recombinant, or mutant. In particular, the plurality of polypeptides may be endogenous to microorganisms, such as bacteria, yeast, or fungi, to a virus, to an animal (including mammals, invertebrates, reptiles, birds, and insects) or to a plant cell.

In addition, the plurality of polypeptides may be obtained from more specific sources, such as the surface coat of a virion particle, a particular cell lysate, a tissue extract, or they may be restricted to those polypeptides that are expressed on the surface of a cell membrane.

Moreover, the plurality of polypeptides may be obtained from a biological fluid, particularly from humans, including but not limited to blood, plasma, serum, urine, feces, mucus, semen, vaginal fluid, amniotic fluid, or cerebrospinal fluid. The plurality of polypeptides may even be obtained from a fermentation broth or a conditioned medium, including all the polypeptide products secreted or produced by the cells previously in the broth or medium.

The step of contacting the recognition unit with the plurality of polypeptides may be effected in a number of ways. For example, one may contemplate immobilizing the recognition unit on a solid support and bringing a solution of the plurality of polypeptides in contact with the immobilized

recognition unit. Such a procedure would be akin to an affinity chromatographic process, with the affinity matrix being comprised of the immobilized recognition unit. The polypeptides having a selective affinity for the recognition unit can then be purified by affinity selection. The nature of the solid support, process for attachment of the recognition unit to the solid support, solvent, and conditions of the affinity isolation or selection procedure would depend on the type of recognition unit in use but would be largely conventional and well known to those of ordinary skill in the art. Moreover, the valency of the recognition unit in the recognition unit complex used to screen the polypeptides is believed to affect the specificity of the screening step, and thus the valency can be chosen as appropriate in view of the desired specificity (see Sections 5.2 and 5.2.1).

Alternatively, one may also separate the plurality of polypeptides into substantially separate fractions comprising individual polypeptides. For instance, one can separate the plurality of polypeptides by gel electrophoresis, column chromatography, or like method known to those of ordinary skill for the separation of polypeptides. The individual polypeptides can also be produced by a transformed host cell in such a way as to be expressed on or about its outer surface. Individual isolates can then be "probed" by the recognition unit, optionally in the presence of an inducer should one be required for expression, to determine if any selective affinity interaction takes place between the recognition unit and the individual clone. Prior to contacting the recognition unit with each fraction comprising individual polypeptides, the polypeptides can optionally first be transferred to a solid support for additional convenience. Such a solid support may simply be a piece of filter membrane, such as one made of nitrocellulose or nylon.

In this manner, positive clones can be identified from a collection of transformed host cells of an expression library, which harbor a DNA construct encoding a polypeptide having a selective affinity for the recognition unit. The

polypeptide produced by the positive clone includes the functional domain of interest or a functional equivalent thereof. Furthermore, the amino acid sequence of the polypeptide having a selective affinity for the recognition unit can be determined directly by conventional means of amino acid sequencing, or the coding sequence of the DNA encoding the polypeptide can frequently be determined more conveniently by use of standard DNA sequencing methods. The primary sequence can then be deduced from the corresponding DNA sequence.

If the amino acid sequence is to be determined from the polypeptide itself, one may use microsequencing techniques. The sequencing technique may include mass spectroscopy.

In certain situations, it may be desirable to wash away any unbound recognition unit from a mixture of the recognition unit and the plurality of polypeptides prior to attempting to determine or to detect the presence of a selective affinity interaction (i.e., the presence of a recognition unit that remains bound after the washing step). Such a wash step may be particularly desirable when the plurality of polypeptides is bound to a solid support.

As can be anticipated, the degree of selective affinities observed varies widely, generally falling in the range of about 1 nM to about 1 mM. In preferred embodiments of the present invention, the selective affinity is on the order of about 10 nM to about 100  $\mu$ M, more preferably on the order of about 100 nM to about 10  $\mu$ M, and most preferably on the order of about 100 nM to about 1  $\mu$ M.

## 5.2. Specificity of Recognition Units

A particular recognition unit may have fairly generic selectivity for a several members (e.g., three or four or more) of a "panel" of polypeptides having the domain of interest (or different versions of the domain of interest or functional equivalents of the domain of interest) or a fairly specific selectivity for only one or two, or possibly three,



of the polypeptides among a "panel" of same. Furthermore, multiple recognition units, each exhibiting a range of selectivities among a "panel" of polypeptides can be used to identify an increasingly comprehensive set of additional  
5 polypeptides that include the functional domain of interest.

Hence, in a population of related polypeptides, the functional domains of interest of each member may be schematically represented by a circle. See, by way of example, Figure 7A. The circle of one polypeptide may overlap  
10 with that of another polypeptide. Such overlaps may be few or numerous for each polypeptide. A particular recognition unit, A, may recognize or interact with a portion of the circle of a given polypeptide which does not overlap with any other circle. Such a recognition unit would be fairly specific to  
15 that polypeptide. On the other hand, a second recognition unit, B, may recognize a region of overlap between two or more polypeptides. Such a recognition unit would consequently be less specific than the recognition unit A and may be characterized as having a more generic specificity depending  
20 on the number of polypeptides that it recognizes or interacts with.

It should also be apparent to those of ordinary skill that any number of B-type recognition units ( $B_1$ ,  $B_2$ ,  $B_3$ , etc.) can be present, each recognizing different "panels" of  
25 polypeptides. Hence, the use of multiple recognition units provides an increasingly more exhaustive population of polypeptides, each of which exhibits a variation or evolution in the functional domain of interest present in the initial target molecule. It should also be apparent to one that the  
30 present method can be applied in an iterative fashion, such that the identification of a particular polypeptide can lead to the choice of another recognition unit. See, e.g., Figure 7B. Use of this new recognition unit will lead, in turn, to the identification of other polypeptides that contain  
35 functional domains of interest that enhance the phenotypic and/or genotypic diversity of the population of "related" polypeptides.

Hence, with a given recognition unit, one may observe interaction with only one or two different polypeptides. With other recognition units, one may find three, four, or more selective interactions. In the situation  
5 in which only a single interaction is observed, it is likely, though not mandatory, that the selective affinity interaction is between the recognition unit and a replica of the initial target molecule (or a molecule very similar structurally and "functionally" to the initial target molecule).

10

5.2.1. Effect of the Presentation of the Recognition Unit Complex on the Specificity of the  
Recognition Unit-Functional Domain Interaction

The present inventors have found, unexpectedly, that the valency (i.e., whether it is a monomer, dimer, tetramer,  
15 etc.) of the recognition unit that is used to screen an expression library or other source of polypeptides apparently has a marked effect upon which genes or polypeptides are identified from the expression library or source of polypeptides. In particular, the specificity of the  
20 recognition unit-functional domain interaction appears to be affected by the valency of the recognition unit in the screening process. By this specificity is meant the selectivity in the functional domains to which the recognition unit will bind in the screening step.

25

As discussed above, in one embodiment, recognition units are obtained by screening a source of recognition units, e.g., a phage display library, for recognition units that bind to a particular target functional domain. Alternatively, database searches for recognition units with sequence homology  
30 to known recognition units can be employed. Of course, if a recognition unit for a particular target functional domain is already known, there is no need to screen a library or other source of recognition units; one can merely synthesize that particular recognition unit. The recognition unit, however  
35 obtained, is then used to screen an expression library or other source of polypeptides, to identify polypeptides that

the recognition unit binds to. A recognition unit that identifies only its target functional domain is a recognition unit that is completely specific. A recognition unit that identifies one or two other polypeptides that do not contain  
5 identically the target functional domain, from among a plurality of polypeptides (e.g., of greater than  $10^4$ ,  $10^6$ , or  $10^8$  complexity), in addition to identifying a molecule comprising its target functional domain, is very or highly specific. A recognition unit that identifies most other  
10 polypeptides present that do not contain its target functional domain, in addition to identifying its target functional domain, is a non-specific recognition unit. In between very specific recognition units and non-specific recognition units, the present inventors have discovered that there are  
15 recognition units that recognize a small number of molecules having functional domains other than their target functional domains. These recognition units are said to have generic specificity.

Thus, there is a "specificity continuum", from  
20 completely and very specific through generic to non-specific, that a recognition unit may evince. See Figure 11 for a depiction of this specificity continuum. The Applicants have discovered that a major factor influencing the specificity exhibited by a recognition unit appears to be the valency of  
25 the recognition unit in the complex used to screen the expression library.

Usually, high specificity is considered to be desirable when screening a library. High specificity is exhibited, e.g., by affinity purified polyclonal antisera  
30 which, in general, are very specific. Monoclonal antibodies are also very specific. Small peptides in monovalent form, on the other hand, generally give very weak, non-specific signals when used to screen a library; thus, they are considered to be non-specific.

35 The present inventors have discovered that recognition units in the form of small peptides, in multivalent form, have a specificity midway between the high

specificity of antibodies and the low/non-specificity of monovalent peptides. Multivalency of the recognition unit of at least two, in a recognition unit complex used to screen the gene library, is preferred, with a multivalency of at least 5 four more preferred, to obtain a screening wherein specificity is eased but not forfeited. In particular, a multivalent (believed to be tetravalent) recognition unit complex comprising streptavidin or avidin (preferably conjugated to a label, e.g., an enzyme such as alkaline phosphatase or 10 horseradish peroxidase, or a fluorogen, e.g. green fluorescent protein) and biotinylated peptide recognition units have an unexpected generic specificity. This allows such peptides to be used to screen libraries to identify classes of polypeptides containing functional domains that are similar 15 but not identical to the peptides' target functional domains. These classes of polypeptides are identified despite the low level of homology at the amino acid level of the functional domains of the members of the classes.

In another specific embodiment, multivalent peptide 20 recognition units may be in the form of multiple antigen peptides (MAP) (Tam, 1989, J. Imm. Meth. 124:53-61; Tam, 1988, Proc. Natl. Acad. Sci. USA 85:5409-5413). In this form, the peptide recognition unit is synthesized on a branching lysyl matrix using solid-phase peptide synthesis methods. 25 Recognition units in the form of MAP may be prepared by methods known in the art (Tam, 1989, J. Imm. Meth. 124:53-61; Tam, 1988, Proc. Natl. Acad. Sci. USA 85:5409-5413), or, for example, by a stepwise solid-phase procedure on MAP resins (Applied Biosystems), utilizing methodology established by the 30 manufacturer. MAP peptides may be synthesized comprising (recognition unit peptide)<sub>2</sub>Lys<sub>1</sub>, (recognition unit peptide)<sub>4</sub>Lys<sub>3</sub>, (recognition unit peptide)<sub>8</sub>Lys<sub>6</sub> or more levels of branching.

The multivalent peptide recognition unit complexes 35 may also be prepared by cross-linking the peptide to a carrier protein, e.g., bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), or an enzyme, by use of known cross-linking

reagents. Such cross-linked peptide recognition units may be detected by, e.g., an antibody to the carrier protein or detection of the enzymatic activity of the carrier protein.

Furthermore, the present inventors have discovered what specificity is exhibited by various types of recognition units and their complexes, i.e., where these recognition units and their complexes fall in the specificity continuum. The present inventors have discovered a range of formats for presenting recognition units used to screen libraries. For example, the present inventors have determined that a peptide in the form of a bivalent fusion protein with alkaline phosphatase is very specific. The same peptide in the form of a fusion protein with the pIII protein of an M13 derived bacteriophage, expressed on the phage surface, has somewhat less, though still high, specificity. That same peptide when biotinylated in the form of a tetravalent streptavidin-alkaline phosphatase complex has generic specificity. Use of such a generically specific peptide permits the identification of a wide range of proteins from expression libraries or other sources of polypeptides, each protein containing an example of a particular functional domain.

Accordingly, the present invention provides a method of modulating the specificity of a peptide such that the peptide can be used as a recognition unit to screen a plurality of polypeptides, thus identifying polypeptides that have a functional domain. In a specific embodiment, specificity is generic so as to provide for the identification of polypeptides having a functional domain that varies in sequence from that of the target functional domain known to bind the recognition unit under conditions of high specificity. In a particular embodiment, the method comprises forming a tetravalent complex of the biotinylated peptide and streptavidin-alkaline phosphatase prior to use for screening an expression library.

35

### 5.3. Kits

The present invention is also directed to an assay kit which can be useful in the screening of drug candidates. In a particular embodiment of the present invention, an assay  
5 kit is contemplated which comprises in one or more containers (a) a polypeptide containing a functional domain of interest; and (b) a recognition unit having a selective affinity for the polypeptide. The kit optionally further comprises a detection means for determining the presence of a polypeptide-  
10 recognition unit interaction or the absence thereof.

In a specific embodiment, either the polypeptide containing the functional domain or the recognition unit is labeled. A wide range of labels can be used to advantage in the present invention, including but not limited to  
15 conjugating the recognition unit to biotin by conventional means. Alternatively, the label may comprise a fluorogen, an enzyme, an epitope, a chromogen, or a radionuclide. Preferably, the biotin is conjugated by covalent attachment to either the polypeptide or the recognition unit. The  
20 polypeptide or, preferably, the recognition unit is immobilized on a solid support. The detection means employed to detect the label will depend on the nature of the label and can be any known in the art, e.g., film to detect a radionuclide; an enzyme substrate that gives rise to a  
25 detectable signal to detect the presence of an enzyme; antibody to detect the presence of an epitope, etc.

A further embodiment of the assay kit of the present invention includes the use of a plurality of polypeptides, each polypeptide containing a functional domain of interest.  
30 The assay kit further comprises at least one recognition unit having a selective affinity for each of the plurality of polypeptides and a detection means for determining the presence of a polypeptide-recognition unit interaction or the absence thereof.

35 A kit is provided that comprises, in one or more containers, a first molecule comprising an SH3 domain and a second molecule that binds to the SH3 domain, i.e., a

recognition unit, where the SH3 domain is a novel SH3 domain identified by the methods of the present invention.

In a specific embodiment, the present invention provides an assay kit comprising in one or more containers:

- 5 (a) a purified polypeptide containing a functional domain of interest, in which the functional domain of is a domain selected from the group consisting of an SH1, SH2, SH3, PH, PTB, LIM, armadillo, Notch/ankyrin repeat, zinc finger, leucine zipper, and helix-turn-helix; and
- 10 (b) a purified recognition unit having a selective binding affinity for said functional domain in said polypeptide.

In the above assay kit, the polypeptide may comprise an amino acid sequence selected from the group consisting of

15 SEQ ID NOS: 8, 10, 12, 18, 20, 22, 24, 30, 32, 38, 40, 190, 192, 194, 196, 198, 200, 221, 113-115, 118-121, 125-128, 133-139, 204-218, and 219.

In the above assay kit, the polypeptide may comprise an amino acid sequence selected from the group consisting of

20 SEQ ID NOS: 6, 14, 16, 26, 28, 34, 36, 112, 116, 117, 122-124, 129-132, and 140.

In other embodiments of the above-described assay kit, the recognition unit may be a peptide. The recognition unit may be labeled with e.g., an enzyme, an epitope, a

25 chromogen, or biotin.

In another specific embodiment, the present invention provides an assay kit comprising in containers:

- (a) a plurality of purified polypeptides, each polypeptide in a separate container and each polypeptide
- 30 containing a functional domain of interest in which the functional domain of interest is a domain selected from the group consisting of an SH1, SH2, SH3, PH, PTB, LIM, armadillo, Notch/ankyrin repeat, zinc fingers, leucine zippers, and helix-turn-helix; and
- 35 (b) at least one recognition unit having a selective binding affinity for said functional domain in each of said plurality of polypeptides.

The present invention also provides an assay kit comprising in one or more containers:

(a) a plurality of purified polypeptides, each polypeptide in a separate container and each polypeptide  
5 containing an SH3 domain; and

(b) at least one peptide having a selective affinity for the SH3 domain in each of said plurality of polypeptides.

The present invention also provides a kit comprising  
10 a plurality of purified polypeptides comprising a functional domain of interest, each polypeptide in a separate container, and each polypeptide having a functional domain of a different sequence but capable of displaying the same binding specificity.

15 In the above-described kits, the polypeptides may have an amino acid sequence selected from the group consisting of: SEQ ID NOs:8, 10, 12, 18, 20, 22, 24, 30, 32, 38, 40, 190, 192, 194, 196, 198, 200, 221.

In the above-described kits, the functional domain  
20 may be an SH3 domain.

The molecular components of the kits are preferably purified.

The kits of the present invention may be used in the methods for identifying new drug candidates and determining  
25 the specificities thereof that are described in Section 5.4.

#### 5.4. Assays for the Identification of Potential Drug Candidates and Determining the Specificity Thereof

The present invention also provides methods for  
30 identifying potential drug candidates (and lead compounds) and determining the specificities thereof. For example, knowing that a polypeptide with a functional domain of interest and a recognition unit, e.g., a binding peptide, exhibit a selective affinity for each other, one may attempt to identify a drug  
35 that can exert an effect on the polypeptide-recognition unit interaction, e.g., either as an agonist or as an antagonist (inhibitor) of the interaction. With this assay, one can



screen a collection of candidate "drugs" for the one exhibiting the most desired characteristic, e.g., the most efficacious in disrupting the interaction or in competing with the recognition unit for binding to the polypeptide.

5           Alternatively, one may utilize the different selectivities that a particular recognition unit may exhibit for different polypeptides bearing the same, similar, or functionally equivalent functional domains. Thus, one may tailor the screen to identify drug candidates that exhibit  
10 more selective activities directed to specific polypeptide-recognition unit interactions, among the "panel" of possibilities. Thus, for example, a drug candidate may be screened to identify the presence or absence of an effect on particular binding interactions, potentially leading to  
15 undesirable side effects.

          Indeed, an intriguing application of the present invention is described as follows. A known antiviral agent, FIAU (a halogenated nucleoside analog), is effective at given dosages against the virus that causes hepatitis B. This  
20 compound is suspected of causing toxic side effects, however, which give rise to liver failure in certain patients to whom the drug is administered. According to the present invention, an assay is provided which can be used to develop a new generation of FIAU-derived drug that maintains its  
25 effectiveness against viral replication while reducing liver toxicity. Such an assay is provided by choosing FIAU as a recognition unit having a selective affinity for a polypeptide present in the hepatitis B virus or a cell infected with the virus. This polypeptide or family of polypeptides having the  
30 functional domain of interest is obtained by allowing the chosen recognition unit, FIAU, to come into contact with an expression library comprised of the hepatitis B virus genome and/or a cDNA expression library of infected cells, according to the methods of the present invention.

35           Likewise, the chosen recognition unit is allowed to come into contact with a plurality of polypeptides obtained from a sample of a human liver extract or of noninfected

hepatocytes. In this manner, a "panel" of polypeptides each of which exhibits a selective affinity for the chosen recognition unit is identified. As described above, this panel is used to determine the activities of drug (FIAU) homologs, analogs, or derivatives in terms of, say, selective inhibition of viral polypeptide-FIAU interaction versus liver polypeptide-FIAU interaction. Hence, those drug homologs, analogs, or derivatives that maintain a selective affinity for the viral polypeptide (or infected cell polypeptide) while failing to interact with or having a minimal binding affinity for liver polypeptides (and, hence, have reduced toxicity in the liver due to elimination of undesirable molecular interactions) can be identified and selected. Additional iterations of this process can be performed if so desired.

Therefore, the present invention contemplates an assay for screening a drug candidate comprising: (a) allowing at least one polypeptide comprising a functional domain of interest to come into contact with at least one recognition unit having a selective affinity for the polypeptide in the presence of an amount of a drug candidate, such that the polypeptide and the recognition unit are capable of interacting when brought into contact with one another in the absence of said drug candidate, and in which the functional domain of interest is a domain selected from the group consisting of an SH1, SH2, SH3, PH, PTB, LIM, armadillo, Notch/ankyrin repeat, zinc finger, leucine zipper, and helix-turn-helix; and (b) determining the effect, if any, of the presence of the amount of the drug candidate on the interaction of the polypeptide with the recognition unit.

In one embodiment, the effect of the drug candidate upon multiple, different interacting polypeptide-recognition unit pairs is determined in which at least some of said polypeptides have a functional domain that differs in sequence but is capable of displaying the same binding specificity as the functional domain in another of said polypeptides.

In another embodiment, at least one of said at least one polypeptide or recognition unit contains a consensus

functional domain and consensus recognition unit,  
respectively.

In another embodiment, the drug candidate is an inhibitor of the polypeptide-recognition unit interaction that  
5 is identified by detecting a decrease in the binding of polypeptide to recognition unit in the presence of such inhibitor.

In another embodiment, said polypeptide is a polypeptide containing an SH3 domain produced by a method  
10 comprising:

- (i) screening a peptide library with an SH3 domain to obtain one or more peptides that bind the SH3 domain;
- (ii) using one of the peptides from step (i) to screen a source of polypeptides to identify one or more  
15 polypeptides containing an SH3 domain;
- (iii) determining the amino acid sequence of the polypeptides identified in step (ii); and
- (iv) producing the one or more novel polypeptides containing an SH3 domain.

20 In another embodiment, said polypeptide is a polypeptide containing an SH3 domain produced by a method comprising:

- (i) screening a peptide library with an SH3 domain to obtain a plurality of peptides that bind the SH3 domain;
- 25 (ii) determining a consensus sequence for the peptides obtained in step (i);
- (iii) producing a peptide comprising the consensus sequence;
- (iv) using the peptide comprising the consensus  
30 sequence to screen a source of polypeptides to identify one or more polypeptides containing an SH3 domain;
- (v) determining the amino acid sequence of the polypeptides identified in step (iv); and
- (vi) producing the one or more polypeptides  
35 containing an SH3 domain.

In a preferred embodiment, the effect of the drug candidate upon multiple, different interacting polypeptide-

recognition unit pairs is determined in which preferably at least some (e.g., at least 2, 3, 4, 5, 7, or 10) of said polypeptides have functional domains that vary in sequence yet are capable of displaying the same binding specificity, i.e., binding to the same recognition unit. In another specific embodiment, at least one of said polypeptides and/or recognition units contain a consensus functional domain and recognition unit, respectively (and thus are not known to be naturally expressed proteins). In one embodiment, the polypeptide is a novel polypeptide identified by the methods of the present invention. In a specific embodiment, an inhibitor of the polypeptide-recognition unit interaction is identified by detecting a decrease in the binding of polypeptide to recognition unit in the presence of such inhibitor.

A common problem in the development of new drugs is that of identifying a single, or a small number, of compounds that possess a desirable characteristic from among a background of a large number of compounds that lack that desired characteristic. This problem arises both in the testing of compounds that are natural products from plant, animal, or microbial sources and in the testing of man-made compounds. Typically, hundreds, or even thousands, of compounds are randomly screened by the use of *in vitro* assays such as those that monitor the compound's effect on some enzymatic activity, its ability to bind to a reference substance such as a receptor or other protein, or its ability to disrupt the binding between a receptor and its ligand.

The compounds which pass this original screening test are known as "lead" compounds. These lead compounds are then put through further testing, including, eventually, *in vivo* testing in animals and humans, from which the promise shown by the lead compounds in the original *in vitro* tests is either confirmed or refuted. See Remington's Pharmaceutical Sciences, 1990, A.R. Gennaro, ed., Chapter 8, pages 60-62, Mack Publishing Co., Easton, PA; Ecker and Crooke, 1995, *Bio/Technology* 13:351-360.

There is a continual need for new compounds to be tested in the *in vitro* assays that make up the first testing step described above. There is also a continual need for new assays by which the pharmacological activities of these  
5 compounds may be tested. It is an object of the present invention to provide such new assays to determine whether a candidate compound is capable of affecting the binding between a polypeptide containing a functional domain and a recognition unit that binds to that functional domain. In particular, it  
10 is an object of the present invention to provide polypeptides, particularly novel ones, containing functional domains and their corresponding recognition units for use in the above-described assays. The use of these polypeptides greatly expands the number of assays that may be used to screen  
15 potential drug candidates for useful pharmacological activities (as well as to identify potential drug candidates that display adverse or undesirable pharmacological activities). In one particular embodiment of the present invention, the polypeptides contain an SH3 domain.

20 In one embodiment of the present invention, such polypeptides are identified by a method comprising: using a recognition unit that is capable of binding to a predetermined functional domain to screen a source of polypeptides, thus identifying novel polypeptides containing the functional  
25 domain or a similar functional domain.

In a particular embodiment of the above-described method, the novel polypeptide comprises an SH3 domain and is obtained by:

(i) screening a peptide library with the SH3 domain  
30 to obtain one or more peptides that bind the SH3 domain;

(ii) using one of the peptides from step (i), preferably in the form of a multivalent complex, to screen a source of polypeptides to identify one or more novel polypeptides containing SH3 domains;

35 (iii) determining the amino acid sequence of the polypeptides identified in step (ii); and

(iv) producing the one or more novel polypeptides containing SH3 domains.

In another embodiment of the above-described method, the novel polypeptide containing an SH3 domain is obtained by:

5 (i) screening a peptide library with the SH3 domain to obtain peptides that bind the SH3 domain;

(ii) determining a consensus sequence for the peptides obtained in step (i);

(iii) producing a peptide comprising the consensus  
10 sequence;

(iv) using the peptide comprising the consensus sequence to screen a source of polypeptides to identify one or more novel polypeptides containing SH3 domains;

(v) determining the amino acid sequence of the novel  
15 polypeptides identified in step (iv); and

(vi) producing the one or more novel polypeptides containing SH3 domains.

One of ordinary skill in the art will recognize that it will not always be necessary to utilize the entire novel  
20 polypeptide containing the SH3 domain in the assays described herein. Often, a portion of the polypeptide that contains the SH3 domain will be sufficient, e.g., a glutathione S-transferase (GST)-SH3 domain fusion protein. See Figure 10A and 10B for a depiction of the portions of the exemplary novel  
25 polypeptides that contain SH3 domains.

A typical assay of the present invention consists of at least the following components: (1) a molecule (e.g., protein or polypeptide) comprising a functional domain; (2) a recognition unit that selectively binds to the functional  
30 domain; (3) a candidate compound, suspected of having the capacity to affect the binding between the protein containing the functional domain and the recognition unit. The assay components may further comprise (4) a means of detecting the binding of the protein comprising the functional domain and  
35 the recognition unit. Such means can be e.g., a detectable label affixed to the protein comprising the functional domain, the recognition unit, or the candidate compound.

In a specific embodiment, the protein comprising the functional domain is a novel protein discovered by the methods of the present invention.

In another specific embodiment, the invention  
5 provides a method of identifying a compound that affects the binding of a molecule comprising a functional domain and a recognition unit that selectively binds to the functional domain comprising:

(a) contacting the molecule comprising the  
10 functional domain and the recognition unit under conditions conducive to binding in the presence of a candidate compound and measuring the amount of binding between the molecule and the recognition unit;

(b) comparing the amount of binding in step (a) with  
15 the amount of binding known or determined to occur between the molecule and the recognition unit in the absence of the candidate compound, where a difference in the amount of binding between step (a) and the amount of binding known or determined to occur between the molecule and the recognition  
20 unit in the absence of the candidate compound indicates that the candidate compound is a compound that affects the binding of the molecule comprising a functional domain and the recognition unit. In a specific embodiment, the molecule comprising the functional domain is a novel protein discovered  
25 by the methods of the present invention. In another specific embodiment, the functional domain is an SH3 domain.

In one embodiment, the assay comprises allowing the polypeptide containing an SH3 domain to contact a recognition unit that selectively binds to the SH3 domain in the presence  
30 and in the absence of the candidate compound under conditions such that binding of the recognition unit to the protein containing an SH3 domain will occur unless that binding is disrupted or prevented by the candidate compound. By detecting the amount of binding of the recognition unit to the  
35 protein containing an SH3 domain in the presence of the candidate compound and comparing that amount of binding to the amount of binding of the recognition unit to the protein or

polypeptide containing an SH3 domain in the absence of the candidate compound, it is possible to determine whether the candidate compound affects the binding and thus is a useful lead compound for the modulation of the activity of proteins containing the SH3 domain. The effect of the candidate compound may be to either increase or decrease the binding.

One version of an assay suitable for use in the present invention comprises binding the protein containing an SH3 domain to a solid support such as the wells of a microtiter plate. The wells contain a suitable buffer and other substances to ensure that conditions in the wells permit the binding of the protein or polypeptide containing an SH3 domain to its recognition unit. The recognition unit and a candidate compound are then added to the wells. The recognition unit is preferably labeled, e.g., it might be biotinylated or labeled with a radioactive moiety, or it might be linked to an enzyme, e.g., alkaline phosphatase. After a suitable period of incubation, the wells are washed to remove any unbound recognition unit and compound. If the candidate compound does not interfere with the binding of the protein or polypeptide containing an SH3 domain to the labeled recognition unit, the labeled recognition unit will bind to the protein or polypeptide containing an SH3 domain in the well. This binding can then be detected. If the candidate compound interferes with the binding of the protein or polypeptide containing an SH3 domain and the labeled recognition unit, label will not be present in the wells, or will be present to a lesser degree than is the case when compared to control wells that contain the protein or polypeptide containing an SH3 domain and the labeled recognition unit but to which no candidate compound is added. Of course, it is possible that the presence of the candidate compound will increase the binding between the protein or polypeptide containing an SH3 domain and the labeled recognition unit. Alternatively, the recognition unit can be affixed to a solid substrate during the assay. Functional



domains other than SH3 domains and their corresponding recognition units can also be used.

In a specific embodiment of the above-described method, the protein or polypeptide containing an SH3 domain is a novel protein or polypeptide containing an SH3 domain that has been identified by the methods of the present invention.

#### 5.5. Use of Polypeptides Containing Functional Domains to Discover Polypeptides Involved in Pharmacological Activities

Using the methods of the present invention, it is possible to identify and isolate large numbers of polypeptides containing functional domains, e.g., SH3 domains. Using these polypeptides, one can construct a matrix relating the polypeptides to an array of candidate drug compounds. For example, Table 1 shows such a matrix.

TABLE 1

	A	B	C	D	E	F	G	H	I	J
1										
2		X		X				X		
3										
4										
5						X				
6										
7			X					X		
8										
9	X									
10										

In Table 1, the columns headed by letters at the top of the table represent different polypeptides containing SH3 domains (preferably novel polypeptides identified by the methods of the invention). The rows numbered along the left side of the table represent recognition units with various specificity to SH3 domains. For each candidate drug compound,

a table such as Table 1 is generated from the results of binding assays. An X placed at the intersection of a particular numbered row and lettered column represents a positive assay for binding, i.e., the candidate drug compound affected the binding of the recognition unit of that particular row to the SH3 domain of that particular column.

Such data as that illustrated above is used to determine whether candidate drug compounds display or are at risk of displaying desirable or undesirable physiological or pharmacological activities. For example, in Table 1, the drug compound inhibits the binding of recognition unit 2 to the SH3 domains of polypeptides B, D, and H; the compound inhibits the binding of recognition unit 5 to the SH3 domain of polypeptide F; the compound inhibits the binding of recognition unit 7 to the SH3 domains of polypeptides C and H; and the compound inhibits the binding of recognition unit 9 to the SH3 domain of polypeptide A.

If interaction with polypeptide H leads to the desirable physiological or pharmacological activity, then this drug candidate might be a good lead. However, interaction with polypeptides A, B, C, D, and F would need to be evaluated for potential side effects.

As the maps are generated and pharmacological effects observed, the maps will allow strategic assessment of the specificity necessary to obtain the desired pharmacological effect. For example, if compounds 2 and 7 are able to affect some pharmacological activity, while compounds 5 and 9 do not affect that activity, then polypeptide H is likely to be involved in that pharmacological activity. For example, if compounds 2 and 7 were both able to inhibit mast cell degranulation, while compounds 5 and 9 did not, it is likely that polypeptide H is involved in mast cell degranulation.

Accordingly, the present invention provides a method of utilizing the polypeptides comprising functional domains of the present invention in an assay to determine the participation of those polypeptides in pharmacological

activities. In a particular embodiment, the polypeptides comprise SH3 domains.

In another embodiment, the method comprises:

(a) contacting a drug candidate with a molecule  
5 comprising a functional domain under conditions conducive to binding, and detecting or measuring any specific binding that occurs; and

(b) repeating step (a) with a plurality of different molecules, each comprising a different functional domain but  
10 capable of binding to a single predetermined recognition unit under appropriate conditions.

Preferably, at least one of said molecules is a novel polypeptide identified by the methods of the present invention. In a specific embodiment, the molecules comprise  
15 the SH3 domains of Src, Abl, Cortactin, Phospholipase C $\gamma$ , Nck, Crk, p53bp2, Amphiphysin, Grb2, RasGap, or Phosphatidylinositol 3' kinase.

The present invention also provides a method of determining the potential pharmacological activities of a  
20 molecule comprising:

(a) contacting the molecule with a compound comprising a functional domain under conditions conducive to binding;

(b) detecting or measuring any specific binding that  
25 occurs; and

(c) repeating steps (a) and (b) with a plurality of different compounds, each compound comprising a functional domain of different sequence but capable of displaying the same binding specificity.

30 In a specific embodiment the functional domain is an SH3 domain.

In another embodiment, the compounds comprise the SH3 domains of Src, Abl, Cortactin, Phospholipase C $\gamma$ , Nck, Crk, p53bp2, Amphiphysin, Grb2, RasGap, or Phosphatidylinositol 3' kinase.  
35

The present invention also provides a method of identifying a compound that affects the binding of a molecule

comprising a functional domain to a recognition unit that selectively binds to the functional domain comprising:

(a) contacting the molecule comprising the functional domain and the recognition unit under conditions conducive to binding in the presence of a candidate compound and measuring the amount of binding between the molecule and the recognition unit and in which the functional domain of interest is a domain selected from the group consisting of an SH1, SH2, SH3, PH, PTB, LIM, armadillo, Notch/ankyrin repeat, zinc finger, leucine zipper, and helix-turn-helix;

(b) comparing the amount of binding in step (a) with the amount of binding known or determined to occur between the molecule and the recognition unit in the absence of the candidate compound, where a difference in the amount of binding between step (a) and the amount of binding known or determined to occur between the molecule and the recognition unit in the absence of the candidate compound indicates that the candidate compound is a compound that affects the binding of the molecule comprising a functional domain and the recognition unit.

In a specific embodiment, the functional domain is an SH3 domain.

#### 5.6. Use of More Than One Recognition Unit Simultaneously

It has been found that when screening a source of polypeptides with a recognition unit, it is possible to use more than one recognition unit at the same time. In particular, it has been found that as many as five different recognition units may be used simultaneously to screen a source of polypeptides.

In particular, when the recognition units are biotinylated peptides and the source of polypeptides is a cDNA expression library, the steps of preconjugation of the biotinylated peptides to streptavidin-alkaline phosphatase as well as the steps involved in screening the cDNA expression library may be carried out in essentially the same manner as is done when a single biotinylated peptide is used as a

recognition unit. See Section 6.1 for details. The key difference when using more than one biotinylated peptide at a time is that the peptides are combined either before or at the step where they are placed in contact with the polypeptides from which selection occurs.

In an embodiment employing a bacteriophage expression library to express the polypeptides, when the positive clones are worked up to the level of isolated plaques, the clonal bacteriophage from the isolated plaques may be tested against each of the biotinylated peptides individually, in order to determine to which of the several peptides that were used as recognition units in the primary screen the phage are actually binding.

#### 5.7. Use of Recognition Units from Known Amino Acid Sequences

In many cases it may not be necessary to screen a collection of substances, e.g., a peptide library, in order to obtain a recognition unit for a given functional domain. In the case of peptide recognition units, for example, it is sometimes possible to identify a recognition unit by inspection of known amino acid sequences. Stretches of these amino acid sequences that resemble known binding sequences for the functional domain can be synthesized and screened against a source of polypeptides in order to obtain a plurality of polypeptides comprising the given functional domain.

Prior to the disclosure of the present invention of methods of preparing recognition units having generic specificity, it would have been thought fruitless to pursue this approach. The expectation would have been that a recognition unit, chosen from published amino acid sequences as described above, would have been useful, at best, to identify a single protein containing a functional domain.

#### 5.8. Isolation and Expression of Nucleic Acids Encoding Polypeptides Comprising a Functional Domain

In particular aspects, the invention provides amino acid sequences of polypeptides comprising functional domains, preferably human polypeptides, and fragments and derivatives thereof which comprise an antigenic determinant (*i.e.*, can be recognized by an antibody) or which are functionally active, as well as nucleic acid sequences encoding the foregoing.

"Functionally active" material as used herein refers to that material displaying one or more functional activities, *e.g.*, a biological activity, antigenicity (capable of binding to an antibody) immunogenicity, or comprising a functional domain that is capable of specific binding to a recognition unit.

In specific embodiments, the invention provides fragments of polypeptides comprising a functional domain consisting of at least 40 amino acids, or of at least 75 amino acids. Nucleic acids encoding the foregoing are provided. Functional fragments of at least 10 or 20 amino acids are also provided.

In other specific embodiments, the invention provides nucleotide sequences and subsequences encoding polypeptides comprising a functional domain, preferably human polypeptides, consisting of at least 25 nucleotides, at least 50 nucleotides, or at least 150 nucleotides. Nucleic acids encoding fragments of the polypeptides comprising a functional domain are provided, as well as nucleic acids complementary to and capable of hybridizing to such nucleic acids. In one embodiment, such a complementary sequence may be complementary to a cDNA sequence encoding a polypeptide comprising a functional domain of at least 25 nucleotides, or of at least 100 nucleotides. In a preferred aspect, the invention utilizes cDNA sequences encoding human polypeptides comprising a functional domain or a portion thereof.

Any eukaryotic cell can potentially serve as the nucleic acid source for the molecular cloning of polypeptides comprising a functional domain. The DNA may be obtained by standard procedures known in the art (*e.g.*, a DNA "library") by cDNA cloning, or by the cloning of genomic DNA, or

fragments thereof, purified from the desired cell (see, for example Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 2d. Ed., Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A  
5 Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the gene encoding a polypeptide comprising a  
10 functional domain should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites  
15 using various restriction enzymes. Alternatively, one may use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to,  
20 agarose and polyacrylamide gel electrophoresis and column chromatography.

Once a gene encoding a particular polypeptide comprising a functional domain has been isolated from a first species, it is a routine matter to isolate the corresponding  
25 gene from another species. Identification of the specific DNA fragment from another species containing the desired gene may be accomplished in a number of ways. For example, if an amount of a portion of a gene or its specific RNA from the first species, or a fragment thereof e.g., the functional  
30 domain, is available and can be purified and labeled, the generated DNA fragments from another species may be screened by nucleic acid hybridization to the labeled probe (Benton, W. and Davis, R., 1977, Science 196, 180; Grunstein, M. And Hogness, D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72, 3961).  
35 Those DNA fragments with substantial homology to the probe will hybridize. In a preferred embodiment, PCR using primers that hybridize to a known sequence of a gene of one species

can be used to amplify the homolog of such gene in a different species. The amplified fragment can then be isolated and inserted into an expression or cloning vector. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further selection can be carried out on the basis of the properties of the gene. Alternatively, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, e.g., has similar or identical electrophoretic migration, isoelectric focusing behavior, proteolytic digestion maps, *in vitro* aggregation activity ("adhesiveness") or antigenic properties as known for the particular polypeptide comprising a functional domain from the first species. If an antibody to that particular polypeptide is available, corresponding polypeptide from another species may be identified by binding of labeled antibody to the putatively polypeptide synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

Genes encoding polypeptides comprising a functional domain can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified DNA of genes encoding polypeptides comprising a functional domain of a first species. Immunoprecipitation analysis or functional assays (e.g., ability to bind to a recognition unit) of the *in vitro* translation products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against polypeptides comprising a functional domain. A radiolabelled cDNA of a



gene encoding a polypeptide comprising a functional domain can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe to identify the DNA fragments that

5 represent the gene encoding the polypeptide comprising a functional domain of another species from among other genomic DNA fragments. In a specific embodiment, human homologs of mouse genes are obtained by methods described above. In various embodiments, the human homolog is hybridizable to the

10 mouse homolog under conditions of low, moderate, or high stringency. By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 h

15 at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml

20 salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10<sup>6</sup> cpm <sup>32</sup>P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is

25 replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art

30 (e.g., as employed for cross-species hybridizations).

By way of example and not limitation, procedures using conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM

35 Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture

containing 100  $\mu\text{g/ml}$  denatured salmon sperm DNA and  $5\text{--}20 \times 10^6$  cpm of  $^{32}\text{P}$ -labeled probe. Washing of filters is done at  $37^\circ\text{C}$  for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC  
5 at  $50^\circ\text{C}$  for 45 min before autoradiography. Other conditions of high stringency which may be used are well known in the art.

The identified and isolated gene encoding a polypeptide comprising a functional domain can then be  
10 inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to,  
15 bacteriophages such as lambda derivatives, or plasmids such as PBR322 or pUC plasmid derivatives. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction  
20 sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically  
25 synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and gene may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation,  
30 etc., so that many copies of the gene sequence are generated.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionization, can be  
35 done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the

isolated gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The nucleic acid coding for a polypeptide comprising a functional domain of the invention can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native gene encoding the polypeptide and/or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a protein or peptide fragment may be regulated by a second nucleic acid sequence so that the protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a protein may be controlled by any promoter/enhancer element known in the art. Promoters which

may be used to control gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290, 304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22, 787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78, 1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296, 39-42); prokaryotic expression vectors such as the  $\beta$ -lactamase promoter (Villa-Komaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75, 3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80, 21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242, 74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303, 209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9, 2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310, 115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38, 639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50, 399-409; MacDonald, 1987, Hepatology 7, 425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315, 115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38, 647-658; Adames et al., 1985, Nature 318, 533-538; Alexander et al., 1987, Mol. Cell. Biol. 7, 1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45, 485-495), albumin gene control region which is

active in liver (Pinkert et al., 1987, Genes and Devel. 1, 268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5, 1639-1648; Hammer et al., 1987, Science 235, 53-58; alpha 1-  
5 antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1, 161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315, 338-340; Kollias et al., 1986, Cell 46, 89-94; myelin basic protein gene control region  
10 which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48, 703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314, 283-286), and gonadotropic releasing hormone gene control region which is active in the  
15 hypothalamus (Mason et al., 1986, Science 234, 1372-1378).

Expression vectors containing inserts of genes encoding polypeptides comprising a functional domain can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene  
20 functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to the inserted gene. In the second approach, the  
25 recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of  
30 foreign genes in the vector. For example, if the gene encoding a polypeptide comprising a functional domain is inserted within the marker gene sequence of the vector, recombinants containing the gene can be identified by the absence of the marker gene function. In the third approach,  
35 recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or

functional properties of the gene product in in vitro assay systems, e.g., ability to bind to recognition units.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

In other specific embodiments, polypeptides comprising a functional domain, or fragments, analogs, or derivatives thereof may be expressed as a fusion, or chimeric protein product (comprising the polypeptide, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence (of a different protein)). Such a chimeric

product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper reading frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer.

#### 5.8.1 Identification and Purification of the Expressed Gene Product

Once a recombinant which expresses the gene sequence encoding a polypeptide comprising a functional domain is identified, the gene product may be analyzed. This can be achieved by assays based on the physical or functional properties of the product, including radioactive labelling of the product followed by analysis by gel electrophoresis.

Once the polypeptide comprising a functional domain is identified, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties may be evaluated using any suitable assay, including, but not limited to, binding to a recognition unit.

#### 5.9 Derivatives and Analogs of Polypeptides Comprising a Functional Domain

The invention further provides derivatives (including but not limited to fragments) and analogs of polypeptides that are functionally active, e.g., comprising a functional domain. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting one or more functional activities associated with a full-length, wild-type polypeptide, e.g., binding to a recognition unit. As one example, such derivatives or analogs may have the antigenicity of the full-length polypeptide.

In particular, derivatives can be made by altering gene sequences encoding polypeptides comprising a functional domain by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a gene encoding a polypeptide comprising a functional domain may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of such genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a polypeptide comprising a functional domain including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Derivatives or analogs of genes encoding polypeptides comprising a functional domain include but are not limited to those polypeptides which are substantially homologous to the genes or fragments thereof, or whose



encoding nucleic acid is capable of hybridizing to a nucleic acid sequence of the genes.

The derivatives and analogs of the invention can be produced by various methods known in the art. The  
5 manipulations which result in their production can occur at the gene or protein level. For example, the cloned gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1989, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold  
10 Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. PCR primers can be constructed so as to introduce desired sequence changes during PCR amplification of  
15 a nucleic acid encoding the desired polypeptide. In the production of the gene encoding a derivative or analog, care should be taken to ensure that the modified gene remains within the same translational reading frame, uninterrupted by translational stop signals, in the gene region where the  
20 desired activity is encoded.

Additionally, the sequence of the genes encoding polypeptides comprising a functional domain can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create  
25 variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978,  
30 J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), etc.

Manipulations of the sequence may also be made at the protein level. Included within the scope of the invention are protein fragments or other derivatives or analogs which are differentially modified during or after translation, e.g.,  
35 by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other

cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease,  $\text{NaBH}_4$ ;

5 acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives can be chemically synthesized. For example, a peptide corresponding to a portion of a polypeptide comprising a functional domain  
10 can be synthesized by use of a peptide synthesizer.

Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino  
15 acids,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine,  $\beta$ -alanine, designer amino acids such as  $\beta$ -methyl amino acids, C $\alpha$ -methyl amino acids, and N $\alpha$ -methyl amino  
20 acids.

#### 5.10 Antibodies to Polypeptides Comprising a Functional Domain

---

According to one embodiment, the invention provides  
25 antibodies and fragments thereof containing the binding domain thereof, directed against polypeptides comprising a functional domain. Accordingly, polypeptides comprising a functional domain, fragments or analogs or derivatives thereof, in particular, may be used as immunogens to generate antibodies  
30 against such polypeptides, fragments or analogs or derivatives. Such antibodies can be polyclonal, monoclonal, chimeric, single chain, Fab fragments, or from an Fab expression library. In a specific embodiment, antibodies specific to the functional domain of a polypeptide comprising  
35 a functional domain may be prepared.

Various procedures known in the art may be used for the production of polyclonal antibodies. In a particular

embodiment, rabbit polyclonal antibodies to an epitope of a polypeptide comprising a functional domain, or a subsequence thereof, can be obtained. For the production of antibody, various host animals can be immunized by injection with the native polypeptide comprising a functional domain, or a synthetic version, or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256, 495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4, 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Antibody fragments which contain the idiotype (binding domain) of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the  $F(ab')$ <sub>2</sub> fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the  $F(ab')$ <sub>2</sub> fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, e.g. ELISA (enzyme-linked immunosorbent assay).

## 6. EXAMPLES

### 6.1. Identification of Genes from cDNA Expression Libraries

5 A study was initiated to determine whether peptide recognition units could recognize functional domains that are the same as or similar to their target functional domain but that are contained in proteins other than the protein containing their target functional domain. Such "functional" screens, using recognition units of relatively small size, 10 were not previously known and were difficult to develop because of the low degree of sequence homology among functional domain-containing proteins. Thus, for example, an oligonucleotide probe could not be designed with any degree of confidence based on the low degree of homology of primary 15 sequences of SH3 domains.

Using SH3 domain-binding peptides from combinatorial peptide libraries as recognition units, we screened a series of mouse and human cDNA expression libraries. We found that 20 69 of the 74 clones isolated from the libraries encoded at least one SH3 domain. These clones represent more than 18 different SH3 domain-containing proteins, of which more than 10 have not been described previously.

The initial recognition unit chosen was a Src SH3 25 domain-binding peptide (termed pSrcCII) isolated from a phage-displayed random peptide library (Sparks et al., 1994, J. Biol. Chem. 269:23853-23856). pSrcCII was (biotin-SGSGGILAPPVPPRNTR-NH<sub>2</sub>) (SEQ ID NO:1). pSrcCII was synthesized by standard Fmoc chemistry, purified by HPLC, and its 30 structure was confirmed by mass spectrometry and amino acid analysis. To form multivalent complexes, 50 pmol biotinylated pSrcCII peptide was incubated with 2 µg streptavidin-alkaline phosphatase (SA-AP) (for a biotin:biotin-binding site ratio of 1:1). Excess biotin-binding sites were blocked by addition of 35 500 pmol biotin. Alternatively, 31.2 µl of 1 mg/ml SA-AP could have been incubated with 15 µl of 0.1 mM biotinylated peptide for 30 min at 4 °C. Ten µl of 0.1 mM biotin would

then be added, and the solution incubated for an additional 15 min.

A  $\lambda$ EX10x mouse 16 day embryo cDNA expression library was obtained from Novagen (Madison, WI). The cDNA library was  
5 screened according to published protocols (Young and Davis, 1983, Proc. Natl. Acad. Sci. USA 80:1194-1198). The library was plated at an initial density of 30,000 plaques/100 mm petri plate as follows. A library aliquot was diluted 1:1000 in SM (100 mM NaCl, 8 mM  $\text{MgSO}_4$ , 50 mM Tris HCl pH 7.5, 0.01%  
10 gelatin). Three  $\mu\text{l}$  of diluted phage were added to 1.5 ml each of SM, 10 mM  $\text{CaCl}_2/\text{MgCl}_2$ , and an overnight culture of BL21(DE3)pLYSE *E. coli* cells. BL21 overnight cultures were grown in 2xYT medium (1.6% tryptone, 1% yeast extract, and 0.5% NaCl) supplemented with 10 mM  $\text{MgSO}_4$ , 0.2 % maltose, and  
15 25 $\mu\text{g}/\text{ml}$  chloramphenicol. This mixture was incubated 20 min at 37°C, after which 300  $\mu\text{l}$  were plated on each of 14 2xYT agar plates in 3 ml 0.8% 2xYT top agarose containing 25  $\mu\text{g}/\text{ml}$  chloramphenicol. Plaques were allowed to form for 6 hours at 37°C, after which isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-  
20 soaked filters were applied. After an additional eight hours' incubation at 37°C, the filters were marked, removed from the plates, and washed three times with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM  $\text{Na}_2\text{HPO}_4$ , 1.4 mM  $\text{KH}_2\text{PO}_4$ ), 0.1% Triton X-100. The filters were blocked for 1 hour in  
25 PBS, 2% bovine serum albumin (blocking solution) and subsequently incubated overnight at 4°C with fresh blocking solution plus streptavidin-alkaline phosphatase (SA-AP) complexed peptide. Approximately 1  $\mu\text{g}$  SA-AP complexed with peptide in 1 ml blocking solution was used for each filter.  
30 The filters were then subjected to four 15 minute washes with PBS, 0.1% Triton X-100. Bound SA-AP-peptide complexes were detected by incubation with 44 ml nitroblue tetrazolium chloride (NBT, 75 mg/ml in 70% dimethylformamide) and 33 ml of 5-bromo-4-chloro-3-indoyl-phosphate-p-toluidine salt (BCIP 50  
35 mg/ml in dimethylformamide) in 10 ml of alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.4, 0.1 M NaCl, 50 mM  $\text{MgCl}_2$ ); the signals were robust, often evident within a few minutes.

Positive plaques were cored with a Pasteur pipet and placed in 1 ml SM with a drop of chloroform. Lambda phage particles are structurally resistant to chloroform, which serves as a bacteriocidal agent. These cores were allowed to diffuse into solution for at least 1 hr before subsequent platings. Phage from cores were plated in 100  $\mu$ l each of SM, 10 mM  $\text{CaCl}_2/\text{MgCl}_2$ , and an overnight culture of BL21 (DE3) pLySE cells. Phage were plated with the intention of reducing the number of plaque forming units (pfu)/plate by roughly a factor of 10 with each screen (i.e.,  $3 \times 10^4$  in the primary screen,  $3 \times 10^3$  in the secondary, and so on). This was accomplished by diluting cores 1:1000 and plating 1-10  $\mu$ l/plate. Four screens were generally required to obtain isolated plaques.

Plasmids were rescued from the  $\lambda$ EXlox phage by cre-mediated excision in BM25.8 *E. coli* cells. For each clone, 5  $\mu$ l of a 1:100 dilution of phage were added to a solution containing 100  $\mu$ l SM and 100  $\mu$ l of an overnight culture of BM25.8 cells (grown in 2xYT media supplemented with 10 mM  $\text{MgSO}_4$ , 0.2 % maltose, 34  $\mu$ g/ml chloramphenicol, and 50  $\mu$ g/ml kanamycin). After 30 minutes at 37  $^\circ\text{C}$ , 100  $\mu$ l of this solution were spread on an LB amp agarose plate and incubated overnight at 37  $^\circ\text{C}$ . A single colony from each plate was used to inoculate 3 ml of 2xYT/amp and incubated overnight. Plasmid DNA was purified from the overnight culture using Promega Wizard Miniprep DNA purification kits (Promega, Madison, WI), extracted with an equal volume of phenol/chloroform followed by chloroform alone, and ethanol precipitated. This plasmid DNA was used to transform chemical-competent DH5 $\alpha$  cells. Three colonies from each transformation were used to inoculate 3 ml cultures; DNA was purified as described above. Approximately, 1/20 of each individually purified DNA sample from transformed cells was digested with EcoRI and HindIII and examined by electrophoresis on a 1% agarose gel to determine insert size and DNA quality. One DNA prep for each clone was either sequenced manually using the dideoxy method or by an automated technique that uses fluorescent dideoxynucleotide terminators.

The T7 gene 10 primer located approximately 40 bp upstream of the EcoRI restriction site was used conveniently in both cases.

Approximately 100 of  $1 \times 10^6$  plaques in the primary  
5 screen of the  $\lambda$ EXlox 16 day mouse embryo cDNA expression  
library exhibited significant pSrcCII-binding activity.  
Figure 5 is representative of filters from primary and  
tertiary screens. Of the eighteen positive clones that were  
isolated and sequenced, all were found to encode proteins with  
10 SH3 domains, although several clones appeared to be siblings  
or to originate from the same mRNA. Thus, the pSrcCII screen  
resulted in the identification of cDNAs encoding nine distinct  
SH3 domain-containing proteins (see Figure 9). The sequences  
of these proteins were compared to the sequences in GenBank  
15 with the computer program BLAST. Three of these proteins  
corresponded to entries in GenBank. SH3P1 appears to be the  
murine homologue of p53bp2, a p53-binding protein, p53bp2  
(Iwabuchi et al., 1994, Proc. Natl. Acad. Sci. USA 91:6098-  
6102); SH3P6 resembles human MLN50, a gene amplified in some  
20 breast carcinomas (Tomasetto et al., 1995, Genomics 28:367-  
376); and SH3P5 is Cortactin, a protein implicated in  
cytoskeletal organization (Wu and Parsons, 1993, J. Cell Biol.  
120:1417-1426). Six of the clones did not match entries in  
GenBank, indicating that the present invention can be used to  
25 identify novel SH3 domain-containing proteins. Of these novel  
proteins, SH3P2 contains three ankyrin repeats and a proline-  
rich region flanking its SH3 domain; SH3P7 and SH3P9 contain  
sequences related to regions in the proteins drebrin (Ishikawa  
et al., 1994, J. Biol. Chem. 269:29928-29933) and amphiphysin  
30 (David et al., 1994, FEBS Lett. 351:73-79), respectively.  
Finally, the novel proteins SH3P4 and SH3P8, although not  
similar to any known proteins, are highly related (89% amino  
acid similarity) to one another.

The present invention can be used as part of an  
35 iterative process in which a recognition unit is used to  
identify proteins containing functional domains which are, in  
turn, used to derive additional recognition units for

subsequent screens. For example, to define the binding specificity of these newly cloned SH3 domains, they can be overexpressed as glutathione S-transferase (GST)-fusion proteins in bacteria, which, in turn, can be used to screen a random peptide library in order to obtain recognition units which, in turn, can be used to screen cDNA libraries in order to obtain still more novel proteins containing SH3 domains.

The recognition unit binding preferences of two of the SH3 domains isolated in the pSrcCII screen described above (p53bp2 and Cortactin) have been described (Sparks et al., 1996, Proc. Natl. Acad. Sci. USA 93:1540-1544. Each of these SH3 domains recognizes recognition unit motifs related to, yet distinct from, the pSrcCII sequence. We used a synthetic peptide (pCort) containing the Cortactin SH3 recognition unit motif to screen the mouse embryo cDNA expression library. pCort was (biotin-SGSGSRLTPQSKPPLPPKPSWVSR-NH<sub>2</sub>) (SEQ ID NO:2). pCort was prepared and complexed with SA-AP as above for pSrcCII. Screening of the mouse embryo library with pCort was done as above for pSrcCII.

Twenty six clones, of varying signal strength, were isolated and twenty-one were found to encode SH3 domain containing proteins. The pCort screen yielded genes corresponding to nine distinct SH3 domain-containing proteins (see Figure 9), four of which corresponded to entries in GenBank. SH3P5 and SH3P6 are Cortactin and MLN50, discussed above; SH3P10 matched SPY75/HS1, a protein involved in IgE signaling (Fukamachi et al., 1994, J. Immunol. 152:642-652); and SH3P11 is Crk, an SH2 domain and SH3 domain-containing adaptor molecule (Knudsen et al., 1994, J. Biol. Chem. 269:32781-32787). The five novel transcripts encode SH3P7, SH3P8, and SH3P9, discussed above; SH3P13, an additional member of the SH3P4/SH3P8 family; and SH3P12, a protein with three SH3 domains and a region sharing significant sequence similarity with the peptide hormone sorbin (Vagen-Descroiz M. et al., 1991, Eur. J. Biochem. 201:53-50).

Interestingly, the output from the pCort screen only partially overlapped with that of the pSrcCII screen: four of



the nine SH3-containing proteins isolated with pCort were not identified with pSrcCII. In addition, SH3P9, the protein identified most frequently (50%) in the pSrcCII screen was isolated at a much lower frequency (7%) with the pCort probe. 5 Thus, different recognition units can be used to identify distinct sets of SH3 domains.

In addition to possessing at least one SH3 domain, a prominent characteristic of the proteins identified in the pSrcCII and pCort screens is the position of the SH3 domain 10 within the proteins: twelve of thirteen proteins possess SH3 domains near their C-termini. Although pSrcCII binds well to the Src SH3 domain (Figure 8), Src (whose SH3 domain occurs near the N-terminus) was not identified in the pSrcCII screen. We suspect the bias was a consequence of the fact that the 15 mouse embryo cDNA library was constructed using oligo-dT-primed cDNA. Alternatively, it may be that the mRNA used to prepare the library contained very little, or no, Src transcripts.

A variant of the pSrcCII peptide (T12SRC.1) was used 20 to probe a  $\lambda$ gt22a human prostate cancer cell line cDNA library primed with oligo-dT and a  $\lambda$ gt11 human bone marrow library primed with random and oligo-dT primers. T12SRC.1 was (biotin-GILAPPVPPRNTR-NH<sub>2</sub>) (SEQ ID NO:3). T12SRC.1 was used in the initial screens together with the peptide T12SRC.4. 25 T12SRC.4 was (biotin-VLKRPLPIPPVTR-NH<sub>2</sub>) (SEQ ID NO:4). The  $\lambda$ gt22a human prostate cancer cell line cDNA library was made from the LNCaP prostate cancer cell line by using standard methods, i.e., the Superscript Lambda system for cDNA synthesis and cloning (Bethesda Research Laboratories, 30 Gaithersburg, MD). The  $\lambda$ gt11 human bone marrow cDNA expression library was obtained from Clonetch (Palo Alto, CA). The human libraries were screened and positive clones isolated as described above for the mouse 16 day embryo cDNA library, except that cDNA inserts of the  $\lambda$ gt11 and  $\lambda$ gt22a phage were 35 amplified by PCR rather than being rescued by cre-mediated excision. Of the  $1.2 \times 10^7$   $\lambda$ cDNA clones screened from these libraries, 30 exhibited detectable pSrcCII-binding activity.

Analysis of the positive clones revealed that they each encoded at least one SH3 domain, and that they originated from a total of six different transcripts (Figure 9). Three of these encode proteins possessing non-C-terminal SH3 domains, 5 indicating that the present invention can be used to identify active domains regardless of their position within a protein. Of the six proteins identified, only three matched GenBank entries. SH3P15 and SH3P16 are Fyn (Kawakami et al., 1988, Proc. Natl. Acad. Sci. USA 85:3870-3874 and Lyn (Yamanashi et 10 al., 1987, Mol. Cell. Biol. 7:237-243), respectively, two Src-family members possessing SH3 domains with ligand preferences similar to that of the Src SH3 domain (Rickles, 1994, EMBO J. 13:5598-5604); and SH3P14 appears to be the human homologue of murine H74, a protein of unknown function. The three 15 remaining proteins did not match entries in GenBank and include the human homolog of SH3P9, described above, and SH3P17 and SH3P18, fragments of two related (85% amino acid similarity) adaptor-like proteins comprised of at least four and three SH3 domains, respectively.

20 Examination of the primary sequences of the SH3 domains identified in this work reveals several interesting features (see Figure 10). Positions important for ligand binding by the Src SH3 domain (Feng et al., 1994, Science 266:1241-1247; Lescure et al., 1992, J. Mol. Biol. 228:387-94) 25 and essential for SH3 function in Grb2/Sem5 are conserved (Clark et al., 1992, Nature 356:340-344). In addition, the two gaps in the sequence alignment shown in Figure 10 correspond to regions of length variation observed among previously characterized SH3 domains. Surprisingly, the SH3 30 domains identified in this work are not significantly more similar to one another than they are to other known SH3 domains, with the exception of the mouse and human forms of SH3P9 and SH3P14 which are 100% and 83% identical, respectively. This result indicates that SH3 domains can vary 35 widely in primary structure and still bind proline-rich peptide recognition units selectively.

6.1.1. Nucleotide and Corresponding Amino Acid  
Sequences of Genes Identified from cDNA  
Expression Libraries

The nucleotide sequences of SH3P1, SH3P2, SH3P3,  
SH3P4, SH3P5, SH3P6, SH3P7, SH3P8, SH3P9, SH3P10, SH3P11,  
5 SH3P12, SH3P13, and SH3P14, the mouse genes identified by  
screening the 16 day mouse embryo cDNA expression library with  
the peptides pSrcII and pCort, are shown in Figures 18, 20,  
22, 24, 26, 28, 30, 32, 34, 38, 40, 42A and B, 44, and 46A and  
B, respectively. The corresponding amino acid sequences of  
10 the mouse genes SH3P1, SH3P2, SH3P3, SH3P4, SH3P5, SH3P6,  
SH3P7, SH3P8, SH3P9, SH3P10, SH3P11, SH3P12, SH3P13, and  
SH3P14 are shown in Figures 19, 21, 23, 25, 27, 29, 31, 33,  
35, 39, 41, 43, 45, and 47, respectively.

The nucleotide sequences of SH3P9, SH3P14, SH3P17,  
15 and SH3P18, human genes identified by screening the human bone  
marrow and human prostate cancer cDNA expression libraries  
with the peptide T12SRC.1, are shown in Figures 36, 48, 50,  
and 52, respectively. The corresponding amino acid sequences  
of the human genes SH3P9, SH3P14, SH3P17, and SH3P18 are shown  
20 in Figures 37, 49, 51, and 53, respectively.

Two genes, SH3P9 and SH3P14, were isolated from both  
mouse and human libraries.

The sequences of SH3P15 and SH3P16 are not shown.  
SH3P15 is Lyn and SH3P16 is Fyn.

25 Figure 54 shows the nucleotide sequence of clone 55,  
a novel human gene identified and isolated from a human bone  
marrow cDNA library (described in Section 6.1) using as  
recognition units a mixture of T12SRC.4 and pCort (described  
in Section 6.1) and the methods described in Section 6.1.

30 Figure 55 shows the amino acid sequence of clone 55.

Figure 56 shows the nucleotide sequence of clone 56,  
a novel human gene identified and isolated from a human bone  
marrow cDNA library (described in Section 6.1) using as  
recognition units a mixture of T12SRC.4 and pCort (described  
35 in Section 6.1) and the methods described in Section 6.1.

Figure 57 shows the amino acid sequence of clone 56.

Figure 58A shows the nucleotide sequence from position 1-1720 and Figure 58B shows the nucleotide sequence from position 1720-2873 of clone 65, a novel human gene identified and isolated from a human bone marrow cDNA library (described in Section 6.1) using as recognition units a mixture of P53BP2.Con and Nck1.Con3 and the methods described in Section 6.1. P53BP2.Con and Nck1.Con3 are peptides, the amino acid sequences of which are biotin-SFAAPARPPVPPRKSRRPGG-NH<sub>2</sub> (SEQ ID NO:201) and biotin-SFSFPPLPPAPGG-NH<sub>2</sub> (SEQ ID NO:202), respectively. The sequences of P53BP2.Con and Nck1.Con3 are consensus sequences of recognition units that bind to the SH3 domains of p53bp2 and Nck, respectively.

Figure 59 shows the amino acid sequence of clone 65.

Figure 60 shows the nucleotide sequence of clone 34, a novel human gene identified and isolated from a human prostate cancer cDNA library (described in Section 6.1) using as recognition units a mixture of T12SRC.1 and T12SRC.4 (described in Section 6.1) and the methods described in Section 6.1.

Figures 61A and 61B show the amino acid sequence of clone 34.

Figure 62 shows the nucleotide sequence of clone 41, a novel human gene identified and isolated from a human bone marrow cDNA library (described in Section 6.1) using as recognition units a mixture of PXXP.NCK.S1/4 and PXXP.ABL.G1/2M and the methods described in Section 6.1. PXXP.NCK.S1/4 and PXXP.ABL.G1/2M are peptides, the amino acid sequences of which are biotin-SRSLSEVSPKPPPIRSVLSLR-NH<sub>2</sub> (SEQ ID NO:222) and biotin-SRPPRWSPPPVPLPTSLSR-NH<sub>2</sub> (SEQ ID NO:223), respectively. PXXP.NCK.S1/4 and PXXP.ABL.G1/2M bind to the SH3 domains of Nck and Abl, respectively.

Figures 63A and 63B show the amino acid sequence of clone 41.

Figure 64 shows the nucleotide sequence of clone 53, a novel human gene identified and isolated from a human prostate cancer cDNA library (described in Section 6.1) using

as recognition units a mixture of PXXP.NCK.S1/4 and PXXP.ABL.G1/2M and the methods described in Section 6.1.

Figures 65A and 65B show the amino acid sequence of clone 53.

5           Figures 66A and 66B show the nucleotide and amino acid sequence of clone 5, a novel human gene identified and isolated from a HELA cell cDNA library using as recognition units a mixture of T12SRC.1 and T12SRC.4 (described in Section 6.1) and the methods described in Section 6.1.

10

#### 6.2. Use of Peptides Resembling SH3 Domain Binding Sequences as Recognition Units

We inspected a number of published amino acid sequences and identified proline-rich stretches of amino acids that resembled consensus SH3 domain binding sequences.  
15           Peptides comprising these proline-rich sequences were synthesized and tested by the methods of the present invention for their ability to specifically bind to the novel SH3 domains described in Sections 6.1 and 6.1.1. Purified SH3  
20           domain-containing clones were spotted on a lawn of Y1090 host cells, grown for an appropriate amount of time, and plaque filter lifts were screened with biotinylated peptides complexed with streptavidin-alkaline phosphatase as described in Section 6.1.

25           The results are shown in Figures 12 and 13. As can be seen, in many cases the synthesized peptides were able to bind to the novel SH3 domains. This indicates that those synthesized peptides could have been used to identify those novel SH3 domains from sources of polypeptides.

30

#### 6.3. Valency of Peptide Recognition Units Affects Specificity of Recognition Units

6.3.1           Preconjugation of Peptide Recognition Units with Streptavidin-Alkaline Phosphatase Increases Affinity of the Recognition Units for  
35           Targets

As a preliminary test of the effect of the valency of peptide recognition units on the ability of those

recognition units to be used as probes to detect SH3 domains, biotinylated peptides that had been previously shown to bind the SH3 domains of either Src or Abl were tested for their ability to bind their respective SH3 domain when either

5 preconjugated with streptavidin-alkaline phosphatase (SA-AP) or not so preconjugated. GST-SrcSH3 and GST-AblSH3 fusion proteins (produced as described in Sparks et al., 1994, J. Biol. Chem. 269:23853-23856) were resolved by 10% SDS-PAGE and transferred to an Immobilon D nylon membranes (Millipore, New

10 Bedford, MA). The membranes were incubated in blocking solution for 1 hr at 25 °C and then incubated overnight at 4 °C with either biotinylated Src SH3 domain or biotinylated Abl SH3 domain binding peptides in either multivalent (SA-AP) or monovalent format. The filters were washed three times (15

15 min each wash) in PBS/T and incubated with NBT and BCIP for color development. See Section 6.1 for further details of the detection process.

The results are shown in Figure 14. In panels A, the biotinylated peptides were preconjugated with SA-AP and

20 then allowed to bind to the immobilized SH3 domains. Preconjugation was as described in Section 6.1. In panels B, the peptides were first allowed to bind to the immobilized SH3 domains and then the bound peptides were detected by adding SA-AP. In both cases, color development was as in Section

25 6.1. The sequences of the peptides used were: Biotin-SGSGGILAPPVPPRNTR (SEQ ID NO:1) for the Src specific peptide and Biotin-SGSGSRPPRWSPPPVPLPTSLDSR (SEQ ID NO:41) for the Abl specific peptide. The results shown in Figure 14 demonstrate that preconjugation with SA-AP dramatically increases the

30 strength of the signal detected.

#### 6.3.2. Preconjugation of Peptide Recognition Units with Streptavidin-Alkaline Phosphatase Results in Recognition of a Variety of SH3 Domains

35 Two µg of each of a panel of GST-SH3 domain fusion proteins were transferred to Immobilon D nylon membranes (Millipore, New Bedford, MA) using a dot-blot apparatus.

Biotinylated Src, Abl, or Cortactin SH3 domain-binding peptides were preconjugated to SA-AP and incubated with the filter; an alkline-phosphatase driven color reaction was used to detect peptide binding. The panel of immobilized proteins was also reacted with a polyclonal anti-GST antibody (Pharmacia, Piscataway, NJ). Sequences of the Src, Abl, and Cortactin-binding peptides were Biotin-SGSGVLKRPLPIPPVTR (SEQ ID NO:42), Biotin-SGSGSRPPRWSPPPVPLPTSLDSR (SEQ ID NO:41), and Biotin-SGSGSRLGEFSKPPIQKPTWMSR (SEQ ID NO:43), respectively.

10 As can be seen from the results shown in Figure 15, the preconjugated biotinylated peptides recognized not only their original target SH3 domains, but related domains as well. The Src peptide recognized the SH3 domains of Yes and Cortactin as well as the SH3 domain of Src; the Abl peptide 15 recognized the Cortactin SH3 domain as well as the Abl SH3 domain; and the Cortactin peptide recognized Src, Yes, Abl, Crk, and the C terminal Grb2 SH3 domains as well as recognizing the Cortactin SH3 domain.

The above experiment was performed utilizing SH3 20 domains that had been immobilized on nylon membranes. The following demonstrates that preconjugation with streptavidin also permits peptide recognition units to recognize a variety of SH3 domains when those domains are immobilized in the wells of a microtiter plate.

25 Five different peptide recognition units (pAbl, pPLC, pCrk, pSrcCI, pSrcCII) were tested in either multivalent or monovalent format for their ability to bind to seven different SH3 domains (Src, Abl, PLC $\gamma$ , Crk, Cortactin, Grb2N, Grb2C) in an ELISA. The sequences of these peptides were as 30 follows: pAbl, SGSGSRPPRWSPPPVPLPTSLDSR (SEQ ID NO:41); pPLC, SGSGSMPPPVPPRPPGTLGG (SEQ ID NO:66); pCrk, SGSGNYVNALPPGPPLPAKNGG (SEQ ID NO:67); pSrcCI, SGSGVLKRPLPIPPVTR (SEQ ID NO:42); pSrcCII, SGSGGILAPPVPPRNTR (SEQ ID NO:1). These peptides were biotinylated as in Section 35 6.1.

The SH3 domains were produced as GST-SH3 fusion proteins as described in Sparks et al., 1994, J. Biol. Chem.

269:23853-23856. Their purity and concentration were confirmed by SDS-PAGE and Bradford protein assays, respectively. The GST-SH3 fusion proteins were immobilized in the wells of microtiter plates as follows: Two micrograms of each GST-SH3 fusion protein were incubated in wells of a flat bottom enzyme linked immunoabsorbent assay (ELISA) microtiter plate (Costar, Cambridge, MA) in 100 mM NaHCO<sub>3</sub> for 1 hr 25 °C. One volume of SuperBlock blocking buffer (Pierce Chemical Co., Rockford, IL) was added to each well and incubated for an additional 30 min. Plates were washed three times with PBS/0.1% Tween-20/0.1% bovine serum albumin (BSA). Immobilized proteins were detected with SH3 domain-binding peptides in multivalent or monovalent formats using streptavidin-horseradish peroxidase (SA-HRP; Sigma Chemical Co., St. Louis, MO). For complexation of the biotinylated peptides and SA-HRP, peptide and SA-HRP concentrations were as described for SA-AP complexation in Section 6.1, but all incubations and washes were in PBS/0.1% Tween-20/0.1% BSA. Plates were washed five times before colorimetric reaction and before the addition of SA-HRP (monovalent format). The amount of bound SA-HRP was evaluated with the addition of 100 µl horseradish peroxidase substrate [2',2'-Azino-Bis 3-Ethylbenzthiazoline-6-Sulfonic Acid (ABTS), 0.05 % hydrogen peroxide, 50 mM sodium citrate, pH 5.0]. After 5-30 minutes of reaction time, the optical densities (OD) of the microtiter plate wells were measured with a microtiter plate scanner (Molecular Devices, Sunnyvale, CA) set for 405 nm wavelength. The results are shown in Figure 8. From Figure 8 it can be seen that the tetravalent (complexed) peptides display both increased affinity and broadened specificity toward SH3 targets. Binding of complexed peptides was, however, still restricted to SH3 domains; the complexes bind to neither GST (Figure 8) nor other unrelated proteins (data not shown). Thus, precomplexation with SA-AP decreases the specificity of the peptide recognition units but does not make the peptides non-specific. Rather, the peptides, when precomplexed,



recognize a variety of SH3 domains in addition to their target domains.

6.3.3. Preconjugation of Peptide Recognition Units with Streptavidin-Alkaline Phosphatase Results in Recognition of a Variety of Expressed cDNA Clones

Lambda phage clones of genes containing a variety of SH3 domains were isolated from screens of a 16 day mouse embryo cDNA expression library (Novagen, Madison, WI). For a description of the isolation of these cDNA clones, see Section 6.1. Phage particles corresponding to individual lambda phage cDNA recombinants were spotted onto 2xYT-1.5 % agar petri plates onto which had been poured 3 ml of 2xYT-0.8 % agarose with 100  $\mu$ l of a BL21(DE3)pLyse *E. coli* culture grown overnight. After a 6 hr incubation at 37 °C, expression of the cDNA segments was induced with IPTG-soaked nitrocellulose filters. After overnight incubation, the expressed proteins had been transferred to the filters and the filters were then incubated with either biotinylated SH3-domain binding peptides preconjugated to SA-AP or a monoclonal antibody recognizing the T7-Tag fusion peptide ( $\alpha$ T7.10Mab; Novagen, Madison, WI). This antibody was used as a positive control since it recognized an epitope expressed by all the clones (part of the  $\phi$ 10 leader sequence common to all  $\lambda$ EXlox recombinants). Sequences of pSrcI, pSrcII, Cortactin, and CaM (Calmodulin binding) peptides were Biotin-SGSGVLKRPLPIPPVTR (SEQ ID NO:42), Biotin-SGSGGILAPPVPPRNTR (SEQ ID NO:1), Biotin-SGSGSRLGEFSKPPIPQKPTWMSR (SEQ ID NO:43), and Biotin-STVPRWIEDSLRGGAARAQTRLASAK (SEQ ID NO:44), respectively.

The results are shown in Figure 16. From Figure 16 it can be seen that precomplexation with SA-AP decreases the specificity of the peptide recognition units but does not make the peptides non-specific; none of the peptides react in a significant fashion with two negative control sequences,  $\alpha$ -actinin and calmodulin (CaM). Rather, the peptides, when precomplexed, recognize a variety of SH3 domain-containing

cDNA clones in addition to clones containing their target domains.

#### 6.4. Characterization of cDNA clone-encoded proteins

##### 5 6.4.1. Production of cDNA clone-encoded proteins

Purified DNA from all positive cDNA clones (ca. 18-20 positive clones per recognition unit) was used to transform chemical-competent BL21 cells (Hanahan et al., 1983, J. Mol. Biol. 166:557-580, the complete disclosure of which is  
10 incorporated by reference herein).

Colonies that appeared after growth overnight at 37 °C on 2xYT agar plates containing 100 µg/ml ampicillin were used to inoculate 4 ml cultures of 2xYT/amp. After 7 hours of incubation at 37 °C with shaking, IPTG was added to each  
15 culture to a final concentration of 100 µM. After an additional 2 hours of incubation, 1 ml of each culture was collected and centrifuged to pellet the cells. Cell pellets were resuspended in 400 µl 1x SDS/DTT loading buffer and boiled at 100 °C for 5 min. The resulting cell lysates were  
20 subjected to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on an 8% acrylamide gel. Gels were either Coomassie stained or transferred to Immobilon D membrane (Millipore) and blotted (Towbin et al., 1979, Proc. Natl. Acad. Sci. 76:4350-4354).

25

#### 6.5. Materials Used in Sections 6.1, 6.2, 6.3.1, 6.3.2, 6.3.3, and 6.4.1

##### **Blocking Solution**

	Hepes (pH 8)	20 mM
30	MgCl <sub>2</sub>	5 mM
	KCl	1 mM
	Dithiothreitol	5 mM
	Milk Powder	5% w/v

##### **2xYT media (1L)**

	Bacto tryptone	16 g
35	Yeast Extract	10 g
	NaCl	5 g

##### **2xYT agar plates**

2xYT + 15 g agar/L

2xYT top agarose (8%)

2xYT + 8 g agarose/L

SDS/DTT loading buffer

5 (10 mL of 5x solution)

.5 M Tris base	0.61 g
8.5% SDS	0.85 g
27.5% sucrose	2.75 g
100 mM DTT	0.154 g
.03% Bromophenol Blue	3.0 mg

- 10 **Overnight cell cultures:**  
Inoculate media with one isolated colony of appropriate cell type and incubate 37 °C O/N with shaking

BL21 (DE3) pLyse

2xYT media

maltose 0.2%

15 MgSO<sub>4</sub> 10 mM

Chloramphenicol 25 µg/mL

BM25.8

2xYT media

maltose 0.2%

MgSO<sub>4</sub> 10 mM

20 Chloramphenicol 34 µg/ml

Kanamycin 50 µg/ml

#### 6.6. Other Functional Domains and Recognition Units

- In a manner similar to that described above for SH3 domains, recognition units directed to other functional domains of interest can be chosen for use in the present method. For example, as recognition units for a study of GST functional domains, the following GST-binding peptides can be used to screen a plurality of polypeptides: Class I CWSEWDGNEC (SEQ ID NO:46), CGQWADDGYC (SEQ ID NO:47), CEOWDGYGAC (SEQ ID NO:48), CWPFWDGSTC (SEQ ID NO:49), CMIWPDGEEC (SEQ ID NO:50), CESOWDGYDC (SEQ ID NO:51), CQQWKEDGWC (SEQ ID NO:52), or CLYOWDGYEC (SEQ ID NO:53); Class II - CMGDNLGDDC (SEQ ID NO:54), CMGDSLGO SC (SEQ ID NO:55), CMDDDLGKGC (SEQ ID NO:56), CMGENLGWSC (SEQ ID NO:57), or CLGESLGWMC (SEQ ID NO:58).

Moreover, the following SH2-binding peptides can be used according to the methods of the present invention to

identify SH2 domain-containing polypeptides: GDGYEEISP (SEQ ID NO:59) (for Src family), GDGYDEPSP (SEQ ID NO:60) (for Nck), GDGYDHPSP (SEQ ID NO:61) (for Crk), GDGYVIPSP (SEQ ID NO:62) (PLC $\gamma$ N), GDGYQNYSP (SEQ ID NO:63) (for PLC $\gamma$ C), GDGYMAMSP (SEQ ID NO:64) (for p85PI3KN and p85PI3KC), or GDGQNYSP (SEQ ID NO:65) (for Grb2). See, Yang, Cell 72:767-778, the complete disclosure of which is incorporated by reference herein.

Further, polypeptides with a "PH" functional domain (analogous to the proteins Vav, Bcr, Msos, PLC $\delta$ , Atk, or Pleckstrin) can be identified using PH-binding peptides, such as those described by Mayer et al., Cell 73:629-630, the complete disclosure of which is incorporated by reference herein.

Other recognition units can be readily contemplated, including other synthetic, semisynthetic, or naturally derived molecules.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

30

35